APPLICATION OF IMMUNOHISTOCHEMISTRY IN THE DIAGNOSIS AND CLASSIFICATION OF ACUTE LEUKEMIA

by

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CERTIFICATE

This is to certify that the dissertation work entitled **"Application of Immunohistochemistry in the Diagnosis and Classification of Acute Leukemia"** submitted by **Dr.S.Priya** is a work done by her during the period of study in this department from 30/05/2010 to 29/05/2013. This work was done under the guidance of **Dr.T.M.SubbaRao,** Professor, Department of Pathology, PSG IMS&R.

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LIST OF COMMONLY USED ABBREVIATIONS

ABBREVIATION	EXPANSION
WHO	World Health Organization
IARC	International Agency For Research On Cancer
AML	Acute Myeloid Leukemia
ALL	Acute Lymphoblastic Leukemia
MDS	Myelodysplastic Syndrome
MPD	Myeloproliferative Disorder
FAB	French American British
MPO	Myeloperoxidase
PAS	Periodic Acid Schiff
TdT	Terminal Deoxynucleotidyl Transferase
CD	Cluster of Differentiation
IHC	Immunohistochemistry
FC	Flow Cytometry

Acute leukemias are characterized by neoplastic proliferation of hematopoietic stem cells and accumulation of blasts and immature cells in the bone marrow. They are broadly classified into two main groups viz., Acute Myeloid Leukemia (AML) and Acute Lymphoid Leukemia (ALL), based on the cellular presentation of the primary stem cell defect. If maturation and differentiation of common myeloid progenitor cell is defective, the leukemia is classified as Acute Myeloid Leukemia and is characterized by clonal expansion of myeloid blasts. On the contrary if the maturation and differentiation of common lymphoid progenitor cell is defective, the leukemia is classified as Acute Lymphoid Leukemia and is characterized by clonal expansion of lymphoid blasts in peripheral blood, bone marrow or other tissues.

The World Health Organization (WHO) further sub classifies these neoplasms based on morphology, cytochemistry, immunophenotyping, cytogenetic and molecular genetic studies.

In our institute, the diagnosis and typing of acute leukemias rested principally on morphological assessment and enzyme cytochemical studies. In a few cases the typing of acute leukemia could be accurately established. However in quite a few cases, the typing of acute leukemia by these two

parameters alone was not possible. A general diagnosis of acute leukemia was offered in such situations. This caused a dilemma to in-house physicians as treatment protocols and prognostic outcomes for AML and ALL are significantly different. Till recently, the gold standard for diagnosis and typing of acute leukemias (that could not classified based on morphology & cytochemistry alone), rested on flow cytometry. This is available in very few select centres and is expensive. Review of recent literature reveals that immunohistochemistry itself can now be used to identify the lineage of leukemic blasts and is an acceptable gold standard. Currently, we are using immunohistochemistry for tissue sections and the department has standardized the protocols for their application on bone marrow trephine biopsy sections. There are many immunohistochemical markers that could be used, but it is important for centres to develop their own panels that would be cost-effective and specific. We therefore, would like to observe, if the application of a specific panel of immunohistochemical markers on the bone marrow trephine tissue sections of acute leukemia, would aid in their diagnosis and typing.

1. To observe the utility of a selective panel of immunohistochemical markers to diagnose and classify acute leukemia.

2. To compare the results of immunohistochemistry with the results of morphology and cytochemistry in acute leukemia.

ETYMOLOGY OF LEUKEMIA AND CLASSIFICATION SYSTEMS:

In 1827, the first case of leukemia was described by a French physician, Alfred-Armand-Louis-Marie Velpeau in a 63-year-old florist with symptoms of fever, weakness, urinary calculi and significant hepatosplenomegaly. He observed that the patient's blood had'gruel' like consistency and speculated that it could be because of the presence of white blood cells. [1]

In 1845, J.H. Bennett, an Edinburgh pathologist described a term 'leucocythemia' to denote a pathological condition in a series of cases, who died with splenomegaly and also had changes in their blood colour and consistency. In 1856, the German pathologist, Rudolf Virchow, coined the term 'leukemia'. It was he who first described that abnormally excess white blood cells were present in blood of patients with symptoms described by Velpeau and Bennett. As he was not certain of the etiology, he used the descriptive term 'leukemia' (Greek; white blood) to refer this condition. In 1878, the term myeloid was introduced by Franz Ernst Christian Neumann. He

was the first person to identify that white blood cells are produced in the bone marrow (Greek; myelos- marrow). [2]

In 1879, Mosler introduced the concept of bone marrow examination for diagnosing leukemia. [3]

In 1891, the technique of staining blood films was described by Paul Ehrlich. He also described the morphology of normal and atypical white blood cells. [4]

In 1889, Wilhelm Ebstein described the term 'acute leukemia' to differentiate rapidly progressive ones which cause immediate death, from, more slowly progressive and indolent chronic leukemias. [5]

In 1900, Otto Naegeli, classified leukemias into two groups, namely, the myeloid and lymphocytic leukemia. He also described that the malignant cell in Acute Myeloid Leukemia (AML), was the myeloblast. [6]

In 1913, leukemias were classified into four main types as chronic lymphocytic leukemia, chronic myelogenous leukemia, acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML). [2]

In 1976, French American British (FAB) co-operative group classification was proposed which sub classified AML into 6 groups (M1 – M6) and ALL into 3 groups (L1 – L3). [7]

In 1981, the FAB classification was revised and a simple scoring system for types L1 and L2 was proposed. [8]

In 1985, another subtype named M7 was added to the FAB classification of AML. [9] In addition to the morphology, immunophenotyping was also used to diagnose this subtype. [10]

In 1991, another subtype M0 was included in the FAB classification of AML. Immunophenotyping, immunocytochemistry and electron microscopy were used to differentiate this subtype from ALL because, the morphology of AML M0 and ALL L2 blasts were very similar. [11]

In 1999, the World Health Organization (WHO) and the International Society of Hematology proposed a new classification of acute leukemias which was published in the year 2001, in the WHO classification of tumors of hematopoietic and lymphoid tissue.

In 2004, a revision of the original 2001 WHO classification was proposed, and it was published in 2008. This classification is followed till date.

In 2008, the genome of AML was fully sequenced. It is the first cancer whose genome is fully sequenced. [12]

DEFINITION OF ACUTE LEUKEMIA – THEN & NOW:

The FAB classification system had defined acute leukemias as neoplasms with presence of 30% or more of blasts in peripheral blood or bone marrow.

The WHO classification of tumors of hematopoietic and lymphoid tissue published in 2001 defined acute myeloid leukemias as neoplasms with presence of $\geq$ 20% myeloblasts in peripheral blood or bone marrow.

There is no agreed upon consensus for acute lymphoblastic leukemias although, treatment centers treated only those with > 25% lymphoblasts in peripheral blood or bone marrow. These criteria have been retained in the most recent WHO classification of tumors of hematopoietic and lymphoid tissue published in 2008. [19]

OVERVIEW ON THE CLASSIFICATION SYSTEMS OF LEUKEMIA:

i) FAB CLASSIFICATION:

A team of seven French, American and British hematologists formed a FRENCH-AMERICAN-BRITISH CO-OPERATIVE GROUP and started working on cases of acute leukemia with the objective of achieving an uniform and consistent system of classification and nomenclature, utilizing the various cell surface antigens that were recently discovered and were thought be characteristic of particular cell types.

The members individually examined Romanowsky stained peripheral blood smears and bone marrow aspirates of 150 patients of acute leukemia. A meeting was held in Paris in October 1974, and the participants discussed the diagnosis made by each one of them and if there was some difference in diagnosis, the slides were reexamined. They worked again on the slides for 8 more months and in July1975, they reached a general agreement and presented the classification after examining some more slides of acute leukemia. [7]

The classification was based on the morphology of predominant cell type in Romanowsky stained smears of peripheral blood and bone marrow which included the size of the cell, nuclear cytoplasmic ratio, presence of granules, and degree of basophilia in the cytoplasm. The morphology was supplemented using cytochemical reactions with myeloperoxidase, Sudan black B and non-specific esterase (naphtholAS- or ASD-acetate) before and after exposure to sodium fluoride.

In this classification, the diagnosis of acute leukemia required the presence of 30% blasts in the blood or bone marrow. [7]

ACUTE LYMPHOBLASTIC LEUKEMIA:

ALL was sub typed into 3 categories based on specific morphological features. They are detailed in table 1.

ALL accounted for <1% of all cancers in adults while in children less than 20 years of age, it comprised 25% of all cancers.

Of the 3 types of ALL, ALL L1 was the most common type and accounted for more than 85% of childhood leukemias.

Table 1: Morphological features of Acute Lymphoblastic Leukemias [7]

Cytological features	L1	L2	L3
Cell size	Small cells predominate	Large, heterogeneous in size	Large and homogeneous
Nuclear chromatin	Homogenous in any one case	Variable-heterogeneous in any one case	Finely stippled and homogeneous
Nuclear shape	Regular,occasional clefting or indendation	Irregular; clefting and indentation common	Regular, oval to round
Nucleoli	Not visible, or small and inconspicuous	One or more present, often large	Prominent; one or more vesicular
Amount of cytoplasm	Scanty	Variable; often moderately abundant	Moderately abundant
Basophilia of cytoplasm	Slight or moderate, rarely intense	Variable; deep in some	Very deep
Cytoplasmic vacuolation	Variable	Variable	Often prominent

ACUTE MYELOID LEUKEMIA:

The FAB cooperative group suggested 6 subtypes of AML as shown in table2.

Table 2: Subtypes of Acute Myeloid Leukaemia – FAB classification. [7]

SUBTYPE	CHARACTERISTICS
M1	Myeloblastic leukemia without maturation
M2	Myeloblastic leukemia with maturation
M3	Hypergranular promyelocytic leukemia
M4	Myelomonocytic leukemia
M5	Monocytic leukemia a – Poorly differentiated (monoblastic) b – Differentiated
M6	Erythroleukemia

Myeloblastic leukemia without maturation (M1):

AML without maturation constituted 5 – 10% of cases of AML. It commonly presented in adults and the average age at presentation was 46

years. Myeloblasts were abundant and comprised more than 90% of the non erythroid cells. [13]

Bone marrow showed few cells (no more than 10%) with granulocytic differentiation. The blasts were homogenous, large, non granular, with occasional blasts showing Auer rods and /or cytoplasmic granules. They had one or more nucleoli. Myeloperoxidase positivity was seen in 3% or more of the blasts.

Myeblastic leukemia with maturation (M2):

This accounted for about 10% of total cases of AML and presented in all age groups. The clinical presentations of the patients were with symptoms related to pancytopenia. [13]

More than 10% cells of the bone marrow showed evidence of granulocytic differentiation and maturation including promyelocytes, myelocytes, metamyelocytes and granulocytes. Blasts accounted for 20 – 89% of non erythroid cells. The blasts had varying amounts of cytoplasm, most of them with granules and had one or more nucleoli. Many blasts contained single Auer rods. The cells could show dysplasia including lobulations in nuclei of myeloblasts, hypogranular myeloblasts and granulocytes could show hypo

or hyper lobated nuclei. Blasts were positive for myeloperoxidase and Sudan black B.

Hypergranular Promyelocytic Leukemia (M3):

5 – 8 % of AML was acute promyelocytic leukemia. This occurred most commonly in adults. Patients could present with features of disseminated intravascular coagulation. [14]

Bone marrow cells were predominantly abnormal promyelocytes with abundant granules in the cytoplasm (hyper granular promyelocytes). The nucleus showed anisopoikilosis and was often bilobed, indented or reniform shaped with one or more nucleoli. The cytoplasm was completely studded with coalescent large granules that obscured the nuclei. The granules were red, deep pink or purple in Romanowsky stained smears. Fine dust like granules could also be found in some cells. The characteristic cells in AML M3 were those with abundant Auer rods that could be present as criss-cross bundles ('Faggot') frequently seen in bone marrow and even in peripheral blood in a few cases.

Myelomonocytic Leukemia (M4):

Acute myelomonocytic leukemia accounted for about 5 – 10% of cases of AML. It more often presented in older age group with an average age at

presentation of 50 years. The patients most often presented with tiredness, fever, anemia, leukocytosis and with low platelet counts. [13]

This type showed proliferation of both monocytic and granulocytic precursors in the bone marrow. The diagnosis required the presence of following features viz., blasts greater than 20%, monocytic component greater than or equal to 20% and granulocytic component greater than or equal to 20% of non-erythroid nucleated cells in the bone marrow and/or peripheral blood monocytic component (monoblasts, promonocytes and monocytes) greater than 5 x 10^9/L. Myeloblasts had varying amounts of cytoplasm, most of them with granules. They had one or more nucleoli and many blasts contained single Auer rods. Monoblasts were usually large with abundant cytoplasm that is bluish gray in colour and showed fine granules, pseudopods or vacuolations. The nucleus was round or convoluted with delicate lacy chromatin and one or more nucleoli. Myeloblasts were myeloperoxidase and Sudan black B positive and monoblasts were naphthol AS-D chloroesterase and alpha- naphthyl acetate esterase positive.

Monocytic Leukemia (M5):

This accounted for less than 5% of all cases of AML. The median age at presentation was 49 years with a slight male predominance. These patients

often had central nervous system, skin and gingival involvement. Extra medullary masses and bleeding tendencies were known to occur frequently in these cases. [13] This type AML was characterized by the presence of greater than or equal to 80% cells of monocytic lineage (monoblasts, promonocytes and monocytes). The granulocytic component was less than 20% of the nucleated cells in the marrow.

There are two subtypes a) poorly differentiated (monoblastic).

b) differentiated.

a) Poorly differentiated subtype was characterized by the presence of abundant large monoblasts in the peripheral blood and bone marrow. The blasts had abundant cytoplasm some with pseudopods (lighter in colour than the rest of cytoplasm) and occasional granules. The nucleus was round or convoluted with delicate lacy chromatin and had prominent and large nucleoli which were also vesicular and varied in number from one to three or more. Only few promonocytes were seen.

b) Differentiated subtype showed all the cells of monocytic series including monoblasts, promonocytes and monocytes. The promonocyte was the predominant cell in the bone marrow and peripheral blood showed a large number of monocytes. The promonocyte had a grayish cytoplasm with a ground glass appearance and fine granules. The nuclei were irregular, large

and convoluted (cerebriform appearance). The diagnosis was confirmed by the cytochemical reactions. These cells were positive for nonspecific esterase and inhibited with fluoride.

Erythroleukemia (M6):

This accounted for < 5% of all cases of AML and occurred primarily in adults. In this type, the erythroblasts and myeloblasts accounted for more than 50% and 20% of bone marrow nucleated cells, respectively. The erythroblasts demonstrated abnormal and bizarre morphology which included nuclear budding, nuclear fragmentation, megaloblastic changes, cytoplasmic vacuoles, karyorrhectic debris, giant multinuclear forms and ringed sideroblasts. The nuclei could be bilobed or multilobated. Erythroblasts could be seen in peripheral blood. Granulocytic cells showed myeloblasts with Auer rods and promyelocytes. Dysmegakaryopoiesis was seen in the form of micromegakaryoblasts and mononuclear megakaryocytes.

Revised FAB classification: First revision - 1981: [8]

The first revision was the result of a review of 300 slides of ALL (done twice) to assess for the concordance in the ALL classification that was based on cell morphology. Based on a series of discussions, the FAB proposed a simpler scoring system for ALL type L1 and ALL type L2. Four

morphological features were the strength of character for this review. They were:

i. Nuclear – cytoplasmic ratio
ii. Nucleolar features such as absence, presence, number and prominence
iii. Nuclear membrane outline (regular or not) and
iv. The size of the cell.

Using this method, there was an increase in the overall concordance (84% from 63%) between the seven observers. There was a drastic difference in the morphological types of ALL between children (≤ 15 years) and adults (≥ 15 years). The most common type of ALL in children was L1 which accounted for 74% of cases, but the most common ALL in adults (66% of cases) was L2. The prognosis of L1 was better than L2 and the latter had a higher relapse rate.

Revised FAB classification: Second revision – 1985: [9]

In 1985, another category called M7 was added by the FAB cooperative study group. The M7 referred to acute leukemia involving the megakaryocyte lineage. The advances in immunophenotyping resulted in the reporting of this type and its subsequent inclusion in the classification system of AML.

A panel of members of South-West oncology group reviewed the slides of acute leukemia to check for the reproducibility of FAB classification of acute leukemia into AML and ALL by morphology and cytochemistry. The various limitations of this classification were analyzed. They proposed that some of the cases of M1, M2 and M4 could be merged into this new category, M7. [9], [15]

Megakaryoblastic leukemia (M7):

This subtype added by the FAB revision in 1985 was rare and accounted for less than 5% cases of AML and could present in both children and adults. In young male patients, a link between acute megakaryoblastic leukemia and occurrence of germ cell tumors in mediastinum was noted. [13]

AML M7 was characterized by the presence of 50% or more blasts of megakaryocytic lineage. These blasts had considerable variation in their size and ranged from to small round cells with basophilic agranular cytoplasm and dense condensed chromatin to large cells with abundant cytoplasm with blebs, with or without granules. The nucleus had single or multiple prominent nucleoli. The blasts could be confirmed as megakaryocytic lineage by immnunophenotyping (CD 41 or CD 61) or by ultrastructural demonstration of platelet peroxidase by electron microscope. The peripheral blood of these patients usually showed pancytopenia or rarely leukocytosis.

Revised FAB classification: Third revision - 1991: [11]

In 1991, the FAB cooperative study group added another new category of AML to their classification called AML- M0 (AML with minimal differentiation). They further stated that AML M0 blasts were very identical to lymphoblasts (most commonly L1 or rarely L2 type) in being large and agranular. Hence, AML M0 myeloblasts and ALL L1/L2 lymphoblasts could not be distinguished by morphology alone. They suggested certain criteria for the diagnosis of AML M0 which are stated below. [11]

- The blasts show negative cytochemical staining for myeloperoxidase
- Staining for Sudan black B should either be negative or if positive, the positivity should be present in less than 3% blasts.
- Immunophenotyping should reveal at least one positive myeloid marker CD 13 or CD33.
- The presence of myeloperoxidase enzyme should be confirmed either by electron microscopy or by immunocytochemistry.
- Immunophenotyping should also confirm the absence of B and T lineage of the blasts.

AML M0:

This subtype represented <5% of all cases of AML and presented at both the extremes of age. The clinical presentation is usually with symptoms related to pancytopenia. [13]

The blasts were of medium size with agranular cytoplasm and the nuclei were either round or had indentations. Nucleoli were present and were variable in number. In most cases, the bone marrow was hypercellular with sheets of undifferentiated blasts. These blasts could not be differentiated from lymphoblasts by morphology or cytochemistry and hence immunophenotyping or ultrastructural studies were mandatory to diagnose this subtype.

ii) WHO CLASSIFICATION - 2001:

World over, acute leukemias were classified based on the FAB system while lymphoid neoplasms were classified based on the Revised European-American Classification of Lymphoid Neoplasms (REAL). There was no standardized classification for other hematopoietic neoplasms such as chronic leukemias, myeloproliferative neoplasms, histiocytic neoplasms etc. The WHO along with the International Society of Hematopathology and European Association of Hematopathology proposed a new classification

addressing these issues. This was a collaborative effort of over 100 pathologists, scientists and clinicians throughout the world. Their clinical and research publications contributed a lot to arrive at this classification. A meeting was held at Airlie, Virginia in USA and cytogenetic abnormalities were added to the classification. [16]

The WHO classification of tumors of hematopoietic and lymphoid tissue which was published in 2001 echoed a paradigm shift in the approach to the classification of hematopoietic neoplasms. Apart from morphology, this classification also incorporated clinical, genetic and immunophenotypic features. The broad headings under which these tumors of the hematopoietic and lymphoid tissues were classified was: [17]

i) Chronic Myeloproliferative Diseases

ii) Myelodysplastic / Myeloproliferative Diseases

iii) Myelodysplastic Syndromes

iv) Acute Myeloid Leukemias

v) B cell neoplasms

vi) T cell and NK cell neoplasms

vii) Hodgkin Lymphoma

viii) Histiocytic and dendritic-cell neoplasms and

ix) Mastocytosis

The salient features of this classification with respect to acute myeloid leukemia are as follows: [17]

- The cut off for blast cells in the peripheral blood or bone marrow for the diagnosis of acute myeloid leukemia was brought down to 20% from the previous 30% requirement in FAB classification.
- Monoblasts and promonocytes in acute monoblastic / monocytic leukemia, megakaryoblasts in acute megakaryoblastic leukemia and promyelocytes in acute promyelocytic leukemia were regarded as 'blast equivalents', while calculating the blast percentage for the diagnosis of AML.
- Erythroblasts were excluded from the blast count except in cases of 'pure' erythroleukemia.
- Another cell excluded from blast count was the dysplastic micromegakaryocyte.
- AML was categorized into 5 subheadings as shown in table 3.

TABLE 3: WHO 2001 classification of AML [17]

I.	*AML WITH RECURRENT GENETIC ABNORMALITIES*
i.	AML with t(8;21)(q22;q22).AML1/ ETO
ii.	AML with abnormal bone marrow eosinophils and (inv 16(p13q22) or t (16; 16) (p13;q22). CBFβ / MYH11
iii.	Acute promyelocytic leukemia with t(15;17)(q22;q12). PML/RARα and variants
iv.	AML with 11q23(MLL) abnormalities
II.	*AML WITH MULTILINEAGE DYSPLASIA*
i.	Following MDS or MDS/MPD
ii.	Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in 2 or more myeloid lineages
III.	*AML AND MDS- THERAPY RELATED*
i.	Alkylating agent / radiation – related type
ii.	Topoisomerase II inhibiter – related type
iii.	Others
IV.	*AML, NOT OTHERWISE CATEGORIZED*
i.	Acute myeloid leukemia, minimally differentiated
ii.	Acute myeloid leukemia without maturation
iii.	Acute myeloid leukemia with maturation
iv.	Acute myelomonocytic leukemia
v.	Acute monoblastic/ acute monocytic leukemia
vi.	Acute erythroid leukemia (erythroid/myeloid and pure erythroleukemia)
vii.	Acute megakaryoblastic leukemia
viii.	Acute basophilic leukemia
ix.	Acute panmyelosis with myelofibrosis
x.	Myeloid sarcoma
V.	*ACUTE LEUKEMIA OF AMBIGUOUS LINEAGE*

The salient features of this classification with respect to acute lymphoblastic leukemia are as follows: [17]

- They are grouped under one major heading 'Precursor B-cell & T-cell neoplasms'.
- The Precursor B-cell neoplasm, also known as Precursor B ALL and the Precursor T-cell neoplasm, also known as Precursor T ALL, were together equivalent to the morphological types L1 & L2 of FAB system.
- Patients with a mass lesion and $\leq$25% blasts in the bone marrow were designated as lymphoblastic lymphoma, while if it was >25%, it could be diagnosed as lymphoblastic leukemia.
- The categorization of ALL as pre-B or pre-T was to be made using immunophenotyping. While both of these blasts were positive for TdT, the former were also positive for CD19 and CD20 while the latter were positive for CD 3 and CD7.

iii) WHO CLASSIFICATION - 2008:

The spectacular advancement in the field of molecular genetics in the past decade, the discovery of various new molecular abnormalities in AML and ALL and increase in targeted gene therapy regimens resulted in revised

classification by the WHO in 2008. This classification is the most recent and in vogue.

Some of the salient changes made are listed below: [18]

- Chronic myeloproliferative diseases were renamed as myeloproliferative neoplasms which included mastocytosis as one of its types.
- A new category of diseases characterized by specific cytogenetic abnormalities and eosinophilia was introduced.
- Two more entities were added onto the list of AML with recurrent genetic abnormalities.
- Acute leukemias of ambiguous lineage was removed from AML category and made an independent entity.
- Precursor lymphoid neoplasms included 3 subcategories. The pre – B was split as those with recurrent genetic abnormalities and those without, the latter called as not otherwise specified (NOS).
- ALL- Burkitt leukemia (L3 of FAB) was placed under mature B-cell neoplasms.

Table 4 lists neoplasms categorized under precursor lymphoid neoplasms

TABLE 4: WHO 2008 CLASSIFICATION OF PRECURSOR LYMPHOID NEOPLASMS

B LYMPHOBLASTIC LEUKEMIA/ LYMPHOMA
A] B lymphoblastic leukemia/ lymphoma, NOS
B] B lymphoblastic leukemia/ lymphoma with recurrent genetic abnormalities
i. B lymphoblastic leukemia/ lymphoma with t(9;22)(q34;q11.2); BCR-ABL1
ii. B lymphoblastic leukemia/ lymphoma with t(v;11q23); MLL rearranged
iii. B lymphoblastic leukemia/ lymphoma with t(12;21)(p13;q22); TEL-AML1 (ETV6 – RUNX1)
iv. B lymphoblastic leukemia/ lymphoma with hyperdiploidy
v. B lymphoblastic leukemia/ lymphoma with hypodiploidy
vi. B lymphoblastic leukemia/ lymphoma with t(5;14)(q31;q32); IL3 – IGH
vii. B lymphoblastic leukemia/ lymphoma with t(1;19)(q23;p13.3); E2A – PBX1 (TCF3 - PBX1)
T LYMPHOBLASTIC LEUKEMIA/ LYMPHOMA

B LYMPHOBLASTIC LEUKEMIA/ LYMPHOMA - NOS:

This represents 80 – 85% of all cases of ALL in children and 70% of cases in adults. The lower limit of lymphoblasts required to make a diagnosis of ALL is not specified, contrary to AML. A diagnosis of ALL was to be avoided if lymphoblasts were <20%. However, the WHO states that a lymphoblast percentage of 25% and above in the bone marrow is used as a cut off for definition of leukemia, in many treatment protocols. [19]. There is no clarity on cases where the lymphoblast percentage was in between these two figures. Patients usually present with symptoms related to bone marrow failure and enlargement of liver and spleen. Lymphoblasts are heterogenous and range from small cells having condensed chromatin and inconspicuous nucleoli to large blasts with grey blue cytoplasm (sometimes vacuolated, 10% may show granules, some show pseudopods imparting a'hand mirror' appearance), dispersed chromatin and many nucleoli. The nuclei may be round or oval, frequently irregular and may show convolutions.

B LYMPHOBLASTIC LEUKEMIA/ LYMPHOMA WITH RECURRENT CYTOGENETIC ABNORMALITIES:

The lymphoblasts listed in this category do not have any morphologic or cytochemical features that are different from those without recurrent genetic

abnormalities. The WHO justifies the need to perform cytogenetic studies in ALL because those that are positive have distinctive clinical properties and prognostic implications.

For instance, those with t (9; 22) (q34; q11.2) have a poor prognosis unless treated with Imatinib. ALL with t (v; 11q23) is the most common leukemia in infants < 1 year of age and has a high frequency of CNS involvement at diagnosis rendering a poor prognosis. More than 90% of ALL children with t (12; 21) or hyperploidy have had complete cures.

T lymphoblastic leukemia/ lymphoma:

This represents 15% of all cases of childhood ALL and 25% of cases in adult population. It occurs more frequently in adolescent males. They usually present as huge mediastinal masses along with hepatosplenomegaly and lymph node enlargement. The lymphoblasts vary in size from medium sized cells with highly condensed chromatin and absent nucleoli to larger blasts which show dispersed chromatin and prominent nucleoli. The nuclei may be round or frequently irregular and some may show convolutions.

DETAILS OF THE WHO 2008 CLASSIFICATION OF ACUTE MYELOID LEUKEMIA:

AML is classified into 6 main groups as shown in table 5. The key changes vis-à-vis the 2001 classification are discussed.

1. AML with recurrent genetic abnormalities

*AML with t(8;21)(q22;q22), AML with inv(16)(p13.1;q22) or t(16;16)(p13.2;q22) and acute promyelocytic leukemia with t(15;17)(q22;12) are considered AML regardless of blast count. Other genetic abnormalities should have a minimum of 20% blasts to be diagnosed as acute leukemia.

*Only cases with t (15;17)(q22;q12) PML – RARA were regarded as Acute promyelocytic leukemia. Cases with variant RARA translocations such as ZBTB16 – RARA, NUMA – RARA, NPM1 – RARA and STAT5B – RARA were recognized separately as they show different morphology such as absent Auer rods, nuclei which are regular and numerous neutrophils with Pelgeroid morphology and strong cytochemical reaction for Myeloperoxidase. Cases with ZBTB16 and STAT5B – RARA fusions showed resistance to treatment with All trans retinoic acid.

Table 5: WHO 2008 classification of AML

1.AML WITH RECURRENT GENETIC ABNORMALITIES	
i.	AML with t(8;21)(q22;q22); RUNX1- RUNX1T1
ii.	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB- MYH11
iii.	Acute promyelocytic leukemia with t(15;17)(q22;q12); PML – RARA
iv.	AML with t(9;11)(p22;q23); MLLT3 – MLL
v.	AML with t(6;9)(p23;q34); DEP – NUP214
vi.	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1 – EVI1
vii.	AML(megakaryoblastic) with t(1;22)(p13;q13); RBM15 – MKL 1
viii.	AML with mutated NPM1
ix.	AML with mutated CEBPA
2. AML WITH MYELODYSPLASIA RELATED CHANGES	
3. THERAPY RELATED MYELOID NEOPLASMS	
4. ACUTE MYELOID LEUKEMIA, NOS	
i.	AML with minimal differentiation
ii.	AML without maturation
iii.	AML with maturation
iv.	Acute myelomonocytic Leukemia
v.	Acute monoblastic and monocytic Leukemia
vi.	Acute erythroid leukemia
vii.	Acute megakaryoblastic leukemia
viii.	Acute basophilic leukemia
ix.	Acute panmyelosis and myelofibrosis
5. MYELOID SARCOMA	
6. MYELOID NEOPLASMS RELATED TO DOWN SYNDROME	
i.	Transient abnormal myelopoiesis
ii.	Myeloid leukemia associated with Down syndrome

* AML with MLL abnormalities was redefined as AML with t (9; 11) (p22; q23); MLLT3 – MLL. Any Abnormalities of MLL other than this translocation should not be included in this category of AML.

* Three new genetic abnormalities were added to the list which includes t(6;9)(p23;q34); DEP – NUP214, inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1 – EVI1 and t(1;22)(p13;q13); RBM15 – MKL 1 to the previous 2001 classification.

* Two new mutations, AML with mutated NPM1 and CEBPA were added as provisional entities.

* It was strongly recommended that cases of AML which do not show any other cytogenetic abnormalities can be tested for Flt3 mutations.

2. AML with myelodysplasia related changes.

* The category AML with multi-lineage dysplasia in previous 2001 classification was renamed as AML with myelodysplasia related changes.

* Certain diagnostic criteria were used to assign AML cases to this category which includes the following:

- Past history of myelodysplastic syndrome which has evolved in to AML.

- Presence of a cytogenetic abnormality related to myelodysplasia.

- If ≥ 2 myeloid cell lineages have ≥ 50% cells with dysplasia

3. Therapy related myeloid neoplasms.

In this group, the sub classifications have been removed.

4. AML, NOS.

* Cases of acute erythroid leukemia which harbor dysplasia are removed from the NOS category and reclassified as AML with myelodysplasia related changes.

* In cases of acute megakaryoblastic leukemia, if any genetic abnormality such as inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1 – EVI1 or t(1;22)(p13;q13); RBM15 – MKL 1 was found, then it should be classified under the suitable category rather under AML, NOS.

* This category should not include cases related to Down syndrome.

5. A new category called myeloid proliferations related to Down syndrome is added.

LABORATORY FINDINGS IN ACUTE LEUKEMIAS:

PERIPHERAL BLOOD SMEAR STUDY:

Examination of a peripheral blood smear stained with May – Grunwald – Giemsa or Wright – Giemsa should be done to evaluate the abnormalities in the red blood cells, white blood cells and platelets. A manual differential count of 200 white blood cells must be done in all cases of AML. [20]

Most of the patients with AML have anemia and thrombocytopenia. Red blood cells can be slightly macrocytic either because they are unable to compete with neoplastic cells for B12 and folic acid or their premature release as reticulocytes. Platelets may be hypogranular and rarely giant platelets can be observed. [21]

The total leucocyte count and the percentage of blasts in AML are quite variable. [22] Neutrophils at times can show signs of dysplasia like hypogranulation, pseudo – pelger – huet anamoly etc. These features of myelodysplasia can be seen in any type of AML but is particularly common in acute promyelocytic leukemia. [23] But the percentage of blasts should be greater than 20% for the diagnosis of acute leukemia. Three different types of neoplastic blasts were defined by the FAB group which includes type I blasts with no granules, type II blasts with less than twenty granules, type III

blasts with abundant granules.[21] Generally a myeloblast has a size of approximately 20µm diameter, round to oval nuclei with dispersed chromatin and prominent nucleoli. Auer rods or granules can be seen in the cytoplasm of some of the blasts.

60 – 70% of patients with ALL at presentation show an increase in the total white blood cell count ranging from 50 to 100 x 10^9/L. Approximately 25% of patients present with a decreased total leucocyte count. [24] There is marked neutropenia in these patients predisposing them to the risk of acquiring various infections. Thrombocytopenia is also present with an average range of 48- 52 x 10^9/L. In children the lymphoblasts are uniform, small and are about two times the size of a small lymphocyte. They have scant to moderate amounts of basophilic cytoplasm and the nucleus is usually round and may show indentations. Nucleoli are generally absent. But in adults, the blast population is heterogeneous with a mixture of smaller and larger lymphoblasts that posses moderate amounts of cytoplasm which is basophilic and may show few granules. Nuclei are irregular with prominent nucleoli. [25]

BONE MARROW ASPIRATE (BMA) SMEAR STUDY:

May – Grunwald – Giemsa or Wright – Giemsa stained, BMA smears should be examined and a perfect area for performing a 500 nucleated cell count should be chosen, which is close to the particle and should not be diluted with blood. If BMA yields a dry tap, then touch imprint can be made from the trephine biopsy core and morphology can be evaluated. However as the differential count on touch imprints will not be representative, it need not be done. [20]

Usually the BMA smears of AML are hypercellular for age. BMA with cellularity of ≤ 30% are referred to as hypoplastic or hypocellular AML. [22]

As defined by the WHO, the blasts should be ≥ 20% of the nucleated non erythroid cells in the bone marrow for the diagnosis of AML to be made. Myeloblasts, monoblasts and megakaryoblasts should be included as blasts for the calculation of the blast count. Promonocytes and abnormal promyelocytes are 'blast equivalents' and should be included in the blast count while calculating the percentage of blasts. Erythroblasts should usually be excluded from the blast count. Only in cases of 'pure' erythroleukemia, erythroblasts are considered as 'blast equivalents' and can be included in the total blast percentage [20]. In 50% of cases, Auer rods can

be seen within the blasts and in few cases the marrow may show an increase in fibrosis. [21]

BMA smears in ALL are hyper cellular for age. Most cases show greater than 65% blasts at presentation. [25] Some lymphoblasts may show intracytoplasmic inclusions which may be of lysosomal origin. [26]

Bone marrow trephine biopsy:

An adequate biopsy should be taken at right angle to the cortical surface. The length of the biopsy should at least be 1.5 cm, as this would contain at least 10 partially preserved inter - trabecular areas for evaluation. The core should be adequately fixed. 3-4µm sections need to be cut and stained with haemotoxylin and eosin. A complete morphological assessment should be made and the following features are to be noted.

1. Overall cellularity of the marrow

2. Arrangement of cells (architecture)

3. Proportion of various haematopoietic cells and their maturation.

4. Stromal changes and fibrosis of the bone marrow.

Cytochemistry:

Cytochemistry is used to identify the chemical composition of blasts by using color reactions and these stains are useful in the identification of blast lineage. They are commonly done on peripheral blood and BMA smears. When correctly performed they can be applied on tissue sections as well. [20]

Myeloperoxidase (MPO) :

Peroxidase enzymes are seen in the granules of myeloid cells. When stained with benzidine chromogens, a positive reaction is indicated by brown coloration of the granules.

All myeloblasts stain positive for MPO except in those of minimally differentiated AML, where the number of positive blasts may be too low to be identified. Monoblasts are generally negative for MPO. However occasional monoblasts and a few promonocytes may show scattered fine positive granules. Megakaryoblasts, lymphoblasts and erythroblasts also show a negative reaction.

Sudan Black B (SBB):

SBB is a lipophilic dye and detects lipids in the granulocytes. The staining reaction is directly proportional to the stage of granulocyte maturation.

When stained using the Sheehan and Storey technique, a positive reaction is black and granular. Myeloblasts stain positive. However the test is less specific than MPO. Rare lymphoblasts are also positive for SBB but the granules stain light grey in color when compared to the myeloblasts which stain deep black in color. [24]

Esterase :

Esterases are esters of carboxylic acids and can hydrolyze the ester bonds in aliphatic and aromatic aminoacids. Esterases with low specificity towards binding the substrate are called non-specific esterases whereas those with high specificity are referred to as specific esterases. These reactions are helpful in distinguishing the cells of neutrophil and monocyte series. [24]

Non specific esterase (NSE):

Two NSE used commonly are α naphthyl butyrate (ANB) and α naphthyl acetate (ANA). The ANB and ANA show diffuse cytoplasmic positivity in monoblasts and also in monocytes. Neutrophils do not stain for NSE and are negative. Erythryoblasts and megakaryoblasts can also show multifocal punctate positive staining reaction for nonspecific esterase but it will be partially resistant to inhibition by sodium fluoride (NaF). But the positive reaction of monocyte and monoblast to NSE can be totally inhibited by

sodium fluoride. Lymphoblasts exhibit focal punctate positivity (golgi zone pattern) to NSE and inhibition by NaF is variable.

Specific esterase:

Cells of neutrophil lineage exhibit positivity for Naphthol – ASD – choloroacetate esterase (CAE). Mast cells are also positive. Neoplastic eosinophils will be positive but normal eosinophils may be negative. Occasional cells in myelomonocytic leukemia will simultaneously stain positive for both specific and non-specific esterases.

Periodic acid Schiff (PAS) :

Carbohydrates with 1, 2 – glycol and glycogen show positivity for PAS stain. In this reaction, the periodic acid is oxidized to dialdehyde in the first step which is then demonstrated by adding Schiff's reagent in the second step. Mature neutrophils (but not myeloblasts), platelets (both mature and immature forms) show strong positivity. Neoplastic proerythroblasts show large globules of PAS positivity and hence can be useful in the diagnosis of acute erythroid leukemia. Lymphoblast exhibits coarse granular or block – like positivity.

Immunophenotyping:

Immunophenotyping is important tool in differentiating AML from ALL. It is also very useful in the immunologic sub classification of ALL into T, B or NK cell type which has important prognostic implications, as the T-cell ALL has a worse prognosis and is resistant to chemotherapy. It is of great advantage in identifying acute leukemias of ambiguous lineage (mixed phenotypic) and in the identification of minimal residual disease.

Immunophenotyping can be done by performing immunohistochemistry on bone marrow trephine biopsy sections or by means of multiparameter flow cytometry. This is based on the fact that different antigens which are expressed on the cells during each stage of normal hematopoiesis are also seen in the neoplasms that develop from the corresponding cells. Both surface antigens markers, monoclonal and polyclonal antibodies are used in the diagnosis. Table 6 shows the various surface antigens expressed on the myeloid lineage cells.

TABLE 6: Antigens expressed by myeloid cells

Name of cell type	Antigen(s) expressed
Hematopoietic stem cell	CD 34
Common myeloid progenitor cell	CD 34, HLA – DR, CD 38
Myeloblast	CD 34, HLA – DR, CD117, CD13, CD33 dim,
Monoblast	CD 34, CD 4, CD 13, CD 33, HLA – DR
Promonocyte	CD 4, CD 13, CD 15, CD33, CD 36, HLA – DR, CD 11b, CD14
Proerythroblast	CD117, CD 36 high, CD 235a low.
Promyelocyte	CD 117, CD 13, CD 33, MPO, CD 65
Megakaryoblast	CD34±, CD 38±, CD 61, CD41

The various antigens expressed on the cell surface of lymphoid cell lineage is shown in table 7.

Table 7: Antigens expressed by lymphoid cells

Name of cell type	Antigen(s) expressed
Lymphoid Progenitor cell	TdT
Pro – B ALL	TdT, cCD 22, CD 34, CD 19
Intermediate pre – B CALL	TdT, CD19, CD 10, CD20±, c CD22
Pre – B ALL	CD19, CD 20, sCD 22, cIg (μ)

Flow cytometry:

Flow cytometry is used to detect antigens on the surface or inside the cell. The cell suspension is passed through a flow chamber and the light scattered by individual cells is detected by a photo detector. In addition, specific

antibodies labeled with fluorescent dyes are used. These antibodies bind the antigen of interest and emit fluorescent light upon excitation. This is detected by means of a photomultiplier tube and finally quantified.

Multiparameter flow cytochemistry is the preferred method for immunophenotyping because of the following advantages. [27]

- In a short time interval, a large number of cells can be analyzed.
- Several antigens can be simultaneously studied on a single neoplastic cell.

Flow cytometry is very useful in differentiating between minimally differentiated AML and ALL and is also used in the subtyping of AML and ALL. The disadvantages are that only fresh and viable cells or tissue can be used and the results cannot be correlated with morphology of cells. [28]

Immunohistochemistry (IHC) on bone marrow trephine biopsy:

Immunophenotyping can also be done by applying immunohistochemical markers to formalin fixed, routinely processed and paraffin embedded bone marrow trephine biopsy tissue. Recently immunohistochemistry is being increasingly used in bone marrow trephine biopsies. [29], [30]

In olden days, the use of IHC was restricted due to the unavailability of good affinity antibodies. At present improvement in techniques to retrieve the antigens and availability of various commercial antibodies have greatly influenced its wide spread use [31]

Principles of IHC: [32]

The tissue antigens are demonstrated by means of antigen antibody interactions. The binding site of antibody can be identified by two methods.

- Direct technique - The primary antibody itself is conjugated to either a flurochrome or an enzyme and this antibody directly binds with the antigen in tissues.
- Two step indirect technique - The primary antibody which is unlabelled binds the antigen in tissues, which in turn is bound by a secondary antibody (labeled with an enzyme and a suitable chromogen substrate).

Advantages:

- It can be performed even on fixed and old archived tissue
- It is particularly important in cases of bone marrow fibrosis, fatty marrow or extremely hypercellular marrow, in which aspirate is very scant or absent (dry tap).

- It is helpful in analyzing extremely fragile cells that cannot withstand the hydrodynamic focusing in flow cytometers. [33]
- Antigens present in the cytoplasm and nucleus of the leukemic cells like myeloperoxidase and terminal deoxynucleotidyl transferase can be readily identified. But these antigens can be detected in flowcytometer only after membrane permeabilization, a difficult step to perform technically.
- Architecture, cellularity and morphology can be assessed.

Disadvantages:

- Longer time required for processing
- Subjective variation in interpretation of results
- Only semiquantitative assessement of tumour cells is possible.
- Only one antibody staining can be done per slide.
- Only select antibodies are available for performing IHC.

Immunohistochemistry can be helpful in distinguishing AML from ALL. Orazi A, in his review article in the Pathobiology journal, listed the various markers used for this purpose. This is furnished in table 8.

Table 8: IHC markers used in distinguishing AML from ALL[23]

AML	**CD34, CD103, MPO, HEMOGLOBIN, CD61, CD42b, CD68R, CD163, LYSOZYME, CD56.**
ALL	**CD34, TdT, CD10, CD79a, PAX5, CD20, CD3, CD7, CD2, CD5, CD7, CD4, CD8, CD1a.**

A wide panel of antibodies is required for immunophenotyping of the subtypes of AML and ALL. Tables 9 & 10 list them based on inputs from the recent edition of the WHO 2008 classification of hematopoietic neoplasms.

Table 9: IHC markers used in subtyping AML [20]

AML with minimal differentiation	**CD34, CD38, HLA – DR, CD33, TdT, CD7.**
AML without maturation	**MPO, CD13, CD33, CD117, CD34,HLA – DR, CD7.**
AML with maturation	**CD13, CD33, CD65, CD11b, CD15, HLA - DR, CD34**
Acute Myelomonocytic Leukemia	**CD13, CD33, CD65, CD15, CD14, CD4, CD11b, CD64, CD36, CD163, LYSOZYME**
Acute monoblastic and monocytic Leukemia	**CD14, CD4, CD11b, CD64, CD36, CD163, LYSOZYME, CD117, HLA – DR**
Acute erythroid Leukemia	**HEMOGLOBIN A, GLYCOPHORIN, CD71(low)**
Acute Megakaryoblastic Leukemia	**CD41, CD61, CD42**

Table 10: IHC markers used in subtyping ALL [19]

B lymphoblastic leukemia/ lymphoma	**CD19, CD79a, CD22, CD10, CD24, PAX5, TdT, CD20, CD34**
T lymphoblastic leukemia/ lymphoma	**CD1a, CD2, CD3, CD4, CD5, CD7, CD8,TdT, CD99, CD34**

OVERVIEW OF THERAPEUTIC STRATEGIES FOR AML:

Over many decades, combination chemotherapy was the standard modality in the treatment of AML. This consisted of an early induction regimen which included cytarabine 100mg/m^2 by continuous infusion for a period of 7 days, followed by 3 days intravenous injections of Daunorubicin. The induction therapy was followed by a consolidation phase which was either chemotherapy with high dose cytarabine for 4 cycles or chemotherapy along with autologous hematopoietic stem cell transplantation or allogenic hematopoietic stem cell transplantation. [34]

With advances in the fields of cytogenetics and molecular genetics, the European Leukemia Network (ELN) has come up with a classification in the

year 2010 which prognosticates leukemic patients into 4 categories as shown in table 11.

The treatment of patients in "favorable" category is same as the standard regimen but the dose of Cytarabine and Daunorubicin could be increased. [35]

Table 11: European Leukemia Network (ELN) prognostic system [36]

Genetic group	Subsets
Favorable	t(8;21)(q22;22), RUNX1 – RUNX1T1, inv(16)(p13.1q22) or t(16;16)(p13.1;q22),CBFB-MYH11 Mutated NPM1 without FLT3 – ITD Mutated CEBPA
Intermediate I	Mutated NPM1 andFLT3 – ITD Wild - type NPM1 and FLT3 – ITD Wild - type NPM1 without FLT3 – ITD
Intermediate II	t(9;11)(p22;q23), MLLT3 – MLL Cytogenetic abnormalities other than favorable or adverse
Adverse	inv(3)(q21;q26.2) or t(3;3)(q21;q26.2), RPN1 – EVI1, t(6;9)(p23;q34), DEK – NUP214, t(V;11)(V;q23), MLL rearranged, - 5 or del(5q), - 7, abnl(17p), complex karyotype.

For treatment of patients in "intermediate" category, newer drugs like nucleoside analogs can be added to Cytarabine. Holowieki suggested the

addition of Cladarabine which resulted in higher cure rates. [37] Faderi et al published that addition of Clofarabine (a newer drug in the same category) in the treatment of elderly AML patients, resulted in higher survival and cure rates. [38]

In patients with FLT3 mutations, drugs with FLT3 inhibition were introduced. Sorafenib is one such drug. [38]

Treatment protocols of AML in the "adverse group" are not clear. While some have advocated standard therapy at higher doses, many studies counter this argument. Many of the patients in this category are being treated with newer molecules as part of drug trials. These molecules include Azacitidine and Decitabine. Preliminary results are not encouraging and many of these patients have been finally treated with Hematopoietic Stem Cell Transplantation [36].

OVERVIEW OF THERAPEUTIC STRATEGIES FOR ALL:

There has been a phenomenal improvement in 5 year survival for pediatric patients with ALL. For instance, it has risen from 3% in 1960 to 84% in the 1996 – 2004 SEER data. The children's oncology group developed a consensus classification to categorize childhood ALL into risk groups. Precursor T – ALL patients are at higher risk than precursor B – ALL.

Children with pre B – ALL were further classified into low and high risk within this subset based on cytogenetic studies. Thus, those children who tested positive for BCR/ ABL and or t (9; 22) were categorized as high risk. Further, children without this cytogenetic abnormality but did not respond to standard induction therapy are also classified as high risk.

Currently ALL in children is treated by a multipronged modality which includes remission induction by vincristine, predsinolone and intrathecal L – asparginase, CNS directed therapy by cranial irradiation, reinduction therapy using methotrexate in addition to the same drugs used for induction of remission and maintenance therapy using daily oral mercaptopurine and weekly oral methotrexate.[39]

Treatment protocols for ALL in adults have been inspired by pediatric regimens. However, treatment outcomes have only improved from 3% in 1960 to 30 – 40% in 1996 – 2004 SEER data. Allogenic stem cell transplantation and newer molecules such as Nelarabine and Clofarabine are being used with increasing success rates. [40]

Before initiating the study, we submitted a protocol of our proposed research work in the prescribed format to the Institute Human Ethics Committee. Our proposal was identified as exempt from review with a note that the researchers need to sign a declaration on confidentiality which we conformed to.

1st January, 2008 to 30th June, 2012 was chosen as our study period. The peripheral smear registers of this period were accessed and cases diagnosed as acute leukemia were noted down. We noted the parameters such as OP / IP number, age, sex, referring unit, diagnosis offered and further tests recommended (if documented). We logged into the 'Patient Result Detail' page of our Hospital Information System using a secured login. Using the OP number as the unique identifier, we traced the case records of all the other investigations performed on each case. We noted the results of the bone marrow aspiration (BMA) and bone marrow trephine (BMT) biopsy studies wherever available. From these sheets we noted the unique numbers allotted to them by the divisions of Clinical Pathology & Histopathology of the Department of Pathology. These numbers are allotted for easy traceability of slides, blocks and reports.

The support staff retrieved all the slides (BMA & BMT) and duplicate copies of the reports. We also procured all the blocks of BMT tissues.

We reviewed all the slides with the reports of results. We paid particular attention to the morphology of the blasts in the peripheral smear and bone marrow aspirate smears. The cytochemistry slides wherever available were also reviewed.

Using a master chart, we made a note of cases where the diagnosis of the type of acute leukemia was made on morphology alone and those that were diagnosed after cytochemistry was performed. We also noted those that bore, just a final diagnosis of acute leukemia with no mention of the type.

Finally we shortlisted all those cases which had all the three diagnostic material needed to complete our study, viz., Peripheral smear, Bone marrow aspiration smear and Bone marrow trephine biopsy specimen and blocks.

Procedure of staining peripheral smears:

All the peripheral smears are stained in the clinical pathology laboratory using commercially prepared Leishman solution and in-house prepared buffer solution (pH 6.8). The staining procedure followed in the laboratory is an adoption of the technique described in the textbook 'Medical Laboratory Science Theory & Practice' by Ochei J & Kolhatkar A. [41]

Briefly, thin smears of peripheral blood were prepared by wedge method, air dried for 10 minutes, stained with Leishman's stain for 2 minutes, diluted with buffered water, mixed well by gentle blowing, left undisturbed for 12 minutes, washed in tap water and dried.

Procedure of staining bone marrow aspirate smears:

Bone marrow aspirations were performed by the treating physicians in the procedure room of the wards. The pediatricians performed it in the minor operation theatre. The material obtained was received by the technician of the clinical pathology lab posted to assist the team. The bone marrow particles were immediately placed on glass slides and, films (approximately 3 – 5 cm in length) were made, as early as possible, with smooth edged spreader slides without delay. Whenever the procedure was followed by a trephine biopsy of the bone marrow, touch imprints were made after which the material was transferred to a pre labeled container, containing 10 ml of B5 fixative.

The bone marrow slides were stained using Giemsa stain. The procedure followed in the lab is based on the Standard Operating Procedures for the Preparation and Staining of Blood Films published online by the Regional Office for South East Asia of the World Health Organization. [42]

The key reagents used were absolute methanol and Giemsa stain [diluted with buffered water- nine volumes and at pH 6.8]. The procedure followed in the CP lab to stain the bone marrow smears is detailed below.

The smears were thoroughly air dried and quickly fixed by immersing in methanol jar for a period of 20 minutes after which they were placed in a jar containing Giemsa stain. The slides were then washed in four changes of water and kept in water subsequently, for 5minutes, for differentiation. The back of the slides were wiped with a clean piece of gauze. Slides were allowed to air dry in a tilted position and finally the slides were mounted. The stained smears were examined under scanner (40x), low power (100x), high power (400x) and oil immersion (1000x) objectives. The reporting format included information on cellularity, erythroid series (including maturation pattern), myeloid series (including an observation on sequential maturation) and megakaryocytes (including nuclear lobations). A 500 cell differential count was done on the cell trails and extrapolated to 100. A detailed description of the morphology and percentage of blasts was observed and documented.

Cytochemistry was performed in many cases. The cytochemical stains used were myeloperoxidase stain and Periodic Acid – Schiff. Esterase stains were not done. The procedures followed in the laboratory are guided by those

described in the textbook authored by Barbara A Brown and titled 'Hematology: Principles & Procedures'. [43]

Myeloperoxidase staining:

Reagents required:

1. Buffered formal acetone (fixative)
2. 3,3' – diaminobenzidine (substrate)
3. Phosphate buffer at pH 7.3 (buffer)
4. Hydrogen peroxide (30%)
5. Aqueous haematoxylin (counterstain)
6. Distrene dibutyl phthalate xylene (DPX) - Mountant

Staining Method:

- Air dried smears were kept in cold buffered formal acetone for 30 seconds. (for fixation)
- The slides were rinsed thoroughly in running tap water and were air dried.
- Slides were incubated for 10 minutes in the working solution. (Mixture of 30 mg of diaminobenzidine in 60 ml of phosphate buffer)
- 120µl of hydrogenperoxide was added and mixed well.

- Slides were counterstained with haemotoxylin for 5 minutes.
- Slides were washed in running tap water.
- The back of the slides was wiped with a clean piece of gauze.
- Slides were allowed to air dry in a tilted position.
- Finally, the slides were mounted.

Interpretation of myeloperoxidase stained smears:

Peroxidase enzyme is present in the granules of myeloid series and to a lesser extent in those of the monocytes. By this technique the granules are stained reddish-brown. Blasts were examined for the presence or absence of these peroxidase staining granules. Other precursors of granulocytes and granulocytes per se served as internal quality controls. In positive cases, the pattern of staining - diffuse cytoplasmic or granular was also noted.

Periodic Acid – Schiff (PAS) staining:

Reagents required:

1. Absolute Methanol (fixative)
2. 1% periodic acid
3. Schiff's reagent
4. Harris haematoxylin (counter stain)

5. Distrene dibutyl phthalate xylene (DPX) - Mountant

Staining method:

- Slides were fixed in absolute methanol for 15 minutes.
- Rinsed in tap water and air dried.
- Slides were flooded with 1% periodic acid for 10 minutes.
- Slides were rinsed in running tap water for 10 minutes and air dried
- Slides were immersed in a coplin jar containing schiff's reagent for 30 minutes with the lid closed.
- Slides were rinsed in running tap water for 10 minutes
- Counterstained with harris haematoxylin for 5 minutes.
- Differentiated in 1% acid alcohol for 10 seconds.
- Washed well in running tap water for 10 minutes.
- The back of the slides were wiped with a clean piece of gauze.
- Slides were allowed to air dry in a tilted position.
- Slides were mounted.

Interpretation of PAS stained smears:

In haemopoietic cells a positive reaction indicates the presence of glycogen in the cytoplasm. The reaction product stains bright magenta to red. Myeloblasts are either negative or show a weak diffuse positivity.

Lymphoblasts may show block positivity. Mature granulocytes whose cytoplasm stained diffusely positive were used as the internal control.

Bone marrow trephine biopsy:

The trephine biopsies were received in B5 solution. After two to four hours of fixation in this solution, the biopsy was fixed subsequently in 10% formalin for at least 12 hours, following which they were decalcified by immersing in a Coplin jar containing 10ml of nitric acid and 100ml of distilled water. The specimens were processed in an automatic tissue processor and embedded in paraffin wax. 3-4µm sections were cut and stained with hematoxylin and eosin. The staining procedure was suited to adjust to the preliminary fixation by B5 as the interfering mercury pigment needed to be removed. [32]

Modified Hematoxylin and eosin (H&E) staining: -

Reagents required:

1. Harris hematoxylin
2. Eosin y 1%
3. Acid alcohol 1% (1% hydrochloric acid in 70% alcohol)
4. Xylene
5. Graded alcohols (70%, 95%, 100%)

6. 3% Lugol's iodine (for removal of mercury pigment of B5 fixative)
7. 2.5% sodium thiosulphate
8. Distrene dibutyl phthalate xylene (DPX) - Mountant

Staining method:

- Sections were deparaffinized by two changes in xylene.
- Hydrated through descending grades of alcohols to water.
- Treated with 3% Lugol's iodine for 5 minutes
- Washed thoroughly in running tap water
- Cleared in 2.5% sodium thio sulphate for 5 minutes
- Washed thoroughly in running tap water
- Stained with Harris haemotoxylin for 5 minutes
- Washed in running tap water for 5 minutes (for blueing of sections)
- Differentiated in 1% acid alcohol for 10 seconds
- Washed in running tap water for 10 minutes, till the sections turned blue again.
- Stained with 1% eosin Y for 2 minutes.
- Washed in running tap water for 5 minutes.
- Dehydrated through ascending grades of alcohols.
- Cleared in xylene.

- Mounted with DPX.

Evaluation of bone marrow trephine biopsy sections stained with H&E:

Reports of the trephine biopsy slides contained information on adequacy (as a general rule, biopsies with at least seven to ten partially preserved inter - trabecular areas were considered as adequate), cellularity for the corresponding age, location and the proportion of normal hematopoietic elements, blasts, stroma etc.

Immunohistochemistry on bone marrow trephine sections:

Immunohistochemistry was performed on the blocks of those cases which had slides and reports of peripheral smear and bone marrow studies, to detect the expression of Myeloperoxidase (MPO), Terminal deoxynucleotidyl transferase (TdT), Cluster of Differentiation 3 (CD3) and Cluster of Differentiation 20 (CD20) in tissue sections by means of the supersensitive polymer – Horse Raddish Peroxidase (HRP) detection system. The general procedure followed is that described in the 1st edition of Diagnostic Immunohistochemistry authored by David J Dabbs. [44] The incubation time was modified as recommended in the kit insert of the manufacturer of the primary antibody (Biogenex).

Reagents used:

1. Primary antibodies available in liquid form (Biogenex, San Ramon, USA):

   * Anti – CD3 (T cell) mouse monoclonal [clone, PS1].

   * Anti – CD20 (B cell) mouse monoclonal [clone, L-26].

   * Anti – Myeloperoxidase (MPO) rabbit polyclonal.

   * Anti – Terminal deoxynucleotidyl transferase (TdT) mouse monoclonal [clone, TdT88].

2. Poly HRP reagent – anti rabbit and anti mouse Immunoglobulin G (IgG) complex linked to Horse Radish Peroxidase enzyme.
3. 3, 3' Diamino benzidine hydrochloride (DAB) – Chromogen.
4. Citrate buffer at pH 6. (2.94 grams of Tri sodium citrate dissolved in 1000ml of distilled water to which 5ml of 1N Hcl was added)
5. Hydrogen peroxide (H2O2) 3% - To block the activity of endogenous peroxidase enzymes.
6. Phosphate buffered saline (PBS), 0.01Molar and at a pH of 7.6. This was prepared in-house as follows:

- Di basic sodium phosphate, anhydrate (Na_2HPo_4) - 17.5 grams.
- Monobasic potassium phosphate, anhydrous (KH_2Po_4) - 2.5 grams.
- Sodium chloride (NaCl) - 17 grams.

- These three substances were dissolved in 1000ml of distilled water.

7. Blocking agent (phosphate buffered saline containing casein and 15 mM sodium azide) – used to block binding of nonspecific proteins.
8. Harris hematoxylin – (for counter staining)
9. Xylene
10. Graded alcohols (70%, 95% and 100%)
11. 3% Lugols iodine (to remove the mercury pigment of B5 fixative)
12. 2.5% Sodium thiosulfate solution
13. Enhancer
14. Distrene dibutyl phthalate xylene (DPX) – mountant.

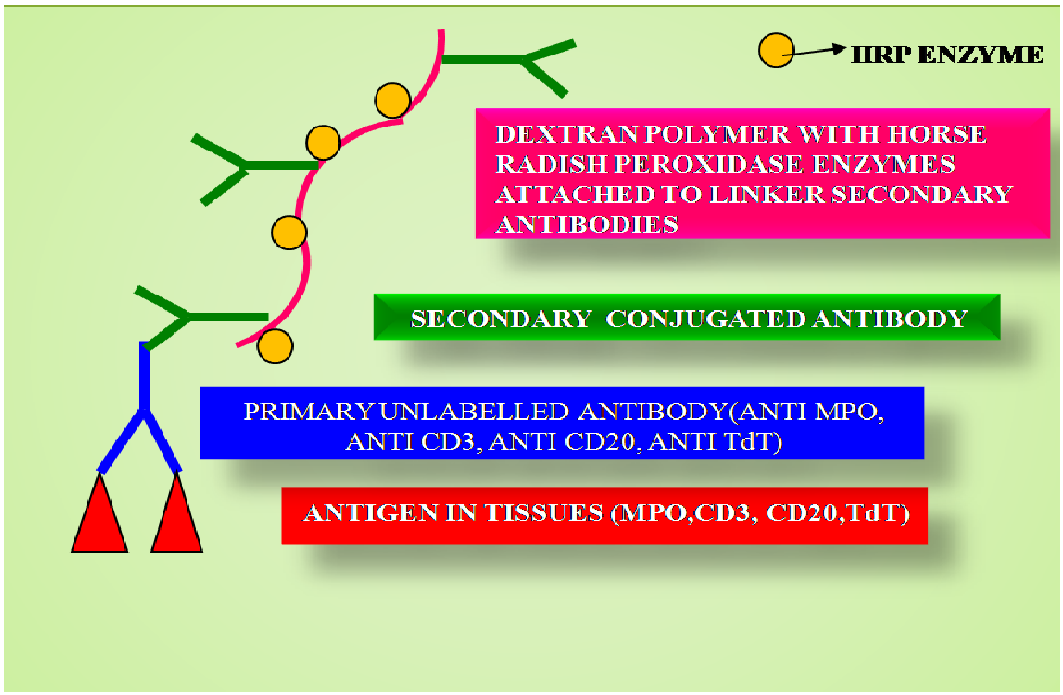
Principle of the detection system:

Antigens present in blasts were detected by a two step process. In the first step, the unlabelled primary antibody (MPO, TdT, CD3 and CD20) against the antigen to be identified was applied. They would bind to the corresponding epitopes present on the blasts.

Next step was the addition of a dextran polymer, containing many molecules of horse radish peroxidase enzyme to which was attached already a linker secondary antibody, directed against the primary antibody.

When a chromogen 3, 3' Diamino benzidine hydrochloride (DAB) was applied, the signal was amplified as several conjugated secondary antibodies reacted with diverse antigenic sites present in the primary antibody.

This principle is illustrated below in a schematic diagram.



Method:

- Paraffin blocks of the trephine biopsies of the study population were taken and fresh sections 4µm in thickness were cut and were floated onto a poly – L – lysine coated slide.
- Paraffin blocks of the following normal tissues (spleen, thymus, tonsils) were chosen and fresh sections 4µm in thickness were cut and

floated on to a poly – L – lysine coated slide to serve as controls for the antibodies (spleen for MPO, thymus for TdT and tonsils for both CD3 & CD20).

- The same procedure was followed for both cases and control slides.
- Slides were incubated overnight in an incubator at a temperature of 37^0C.
- Then slides were deparaffinized in two changes of xylene 10 minutes each.
- Rehydrated through descending grades of alcohols for 5minutes in each.
- Treated with 3% Lugol's iodine for 5 minutes.
- Washed in running tap water.
- Cleared with 2.5% sodium thio sulphate solution for 5 minutes.
- Rehydrated by washing well in running tap water for 5 minutes.
- Heat mediated antigen retrieval was done using a pressure cooker at 95^0C, for 10 minutes.
- The cooker was kept inside sink with water for 20 minutes and brought to room temperature.
- Slides were washed in PBS buffer at pH 7.6 twice, each wash for 5 minutes.

- To block the endogenous peroxidase activity, the slides were immersed in 0.3% hydrogen peroxide for a period of 20 minutes.
- Slides were washed in PBS buffer at pH 7.6 thrice, each wash for 5 minutes.
- To block nonspecific protein binding, the slides were incubated for 10 minutes with 100µl of blocking solution (Power block, Biogenex).
- Slides were washed in PBS buffer at pH 7.6 thrice, each wash for 5 minutes.
- Then slides were incubated with 100µl of primary antibodies (MPO, TdT, CD20 and CD3, Biogenex) on the respective sections. The incubation period was as follows;

  MPO – 30 minutes at room temperature

  CD20 – 30 minutes at room temperature

  CD3 – 1 hour at room temperature

  TdT – 2 hours at room temperature

- To increase the signal intensity, the slides were incubated with 100µl of an enhancer (Super enhancer, Biogenex) for 20 minutes.
- Slides were washed in PBS buffer at pH 7.6 thrice, each wash for 5 minutes.

- Slides were incubated with 100µl of polymer HRP (Horse radish peroxidase reagent, Biogenex) for 30 minutes.
- Slides were washed in PBS buffer at pH 7.6 thrice, each wash for 5 minutes.
- Diamino benzidine (DAB) was freshly prepared by adding 1 drop of liquid DAB chromogen to 1 ml of stable DAB buffer. 100µl of this was added to the slides and incubated at room temperature 5 minutes.
- Slides were washed well in running tap water.
- Counterstained with Harris haemotoxylin for 3 minutes.
- Washed well in running tap water.
- Dehydrated in ascending grades of alcohols for 5 minutes each.
- Sections were cleared in two changes of xylene, 10 minutes each.
- Mounted with Distrene dibutyl phthalate xylene (DPX).

Evaluation of IHC staining:

The slides were examined systematically under scanner (4x), low power (100x) and high power (400x) objectives. A well stained area without any nonspecific background staining was chosen to assess the antigen expression of the blasts. The reaction was considered positive if the following pattern of staining was observed in more than 10% of the neoplastic cells. [45]

MPO – brown colored cytoplasmic staining.

TdT – brown colored nuclear staining.

CD20 – brown colored membranous accentuation and some cytoplasmic staining.

CD3 – brown colored membranous accentuation.

The observations of Immunohistochemistry studies were documented in the master chart and results analyzed.

During the study period between 1st January, 2008 and 30th June, 2012, the Clinical Pathology Laboratory (C.P. Lab) of PSG hospitals performed about eleven lakh tests. 58% of these were hematology tests while the remainder was composed of tests performed on urine, body fluids, stool, semen etc. Table 12 shows the breakup of the total tests performed in the C.P. Lab during the study period.

Table 12: Break up of tests performed by the CP Lab during the study period.

Broad category	No of tests performed	% of total tests
Hematology tests	6,41,803	58%
Non – hematology tests	4,56,747	42%
Total tests performed	10,98,550	

46,227 of the hematology tests were peripheral smears. During the same period, 1088 bone marrow aspiration tests were performed. The remainder of the tests was Complete Blood Counts, Prothrombin Time, Activated Partial Thromboplastin Time, Reticulocyte count, ESR and isolated blood count requests such as Total WBC count, Hemoglobin, Hematocrit, Platelet count etc.

Table 13 shows the breakup of the hematological tests performed during the study period. Peripheral smears accounted for 7% of the total tests while bone marrow aspirations accounted for less than 0.2% tests of the total hematological tests.

Table 13: Breakup of types of hematological tests performed by the CP Lab during the study period

Test item	Number of tests	% of total tests
Peripheral smear study	46,227	7%
BMA smear study	1,088	0.16%
Other tests such as CBC, PT, APTT, ESR etc	5,94,488	92.84%
Total hematology tests	6,41,803	

Of the 46,227 peripheral smears and 1088 bone marrow aspiration smears, 344 cases were diagnosed as leukemia. Table 14 depicts the breakup of the types of leukemias reported during the study period.

Table 14: Types of leukemias reported during the study period

S.No	Diagnostic category	No. of cases
1.	Acute leukemia	99
2.	Chronic myeloid leukemia	130
3.	Chronic lymphocytic leukemia	114
4.	Chronic myelomonocytic leukemia	1
5.	Total	344

Thus acute leukemias comprise 28.7% of the total leukemias reported during the study period.

There was a bimodal peak age of occurrence i.e., 0-20 years and 41-60 years. The mean age was 39 years. Figure 1 shows the age distribution of the reported cases of acute leukemia.

Figure 1: Age distribution of acute leukemia

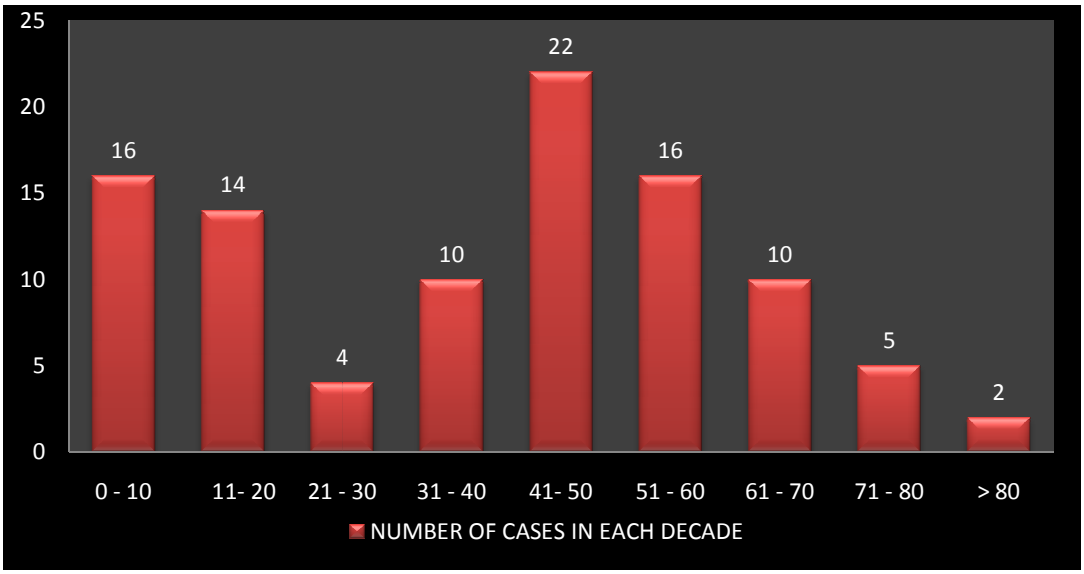
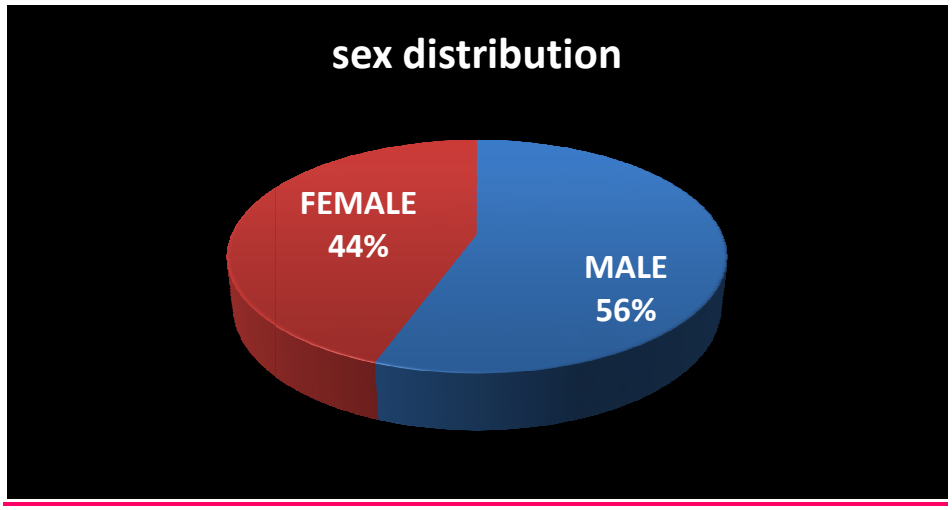


Figure 2 shows the sex distribution of acute leukemia reported during the study period. Males slightly outnumber females.

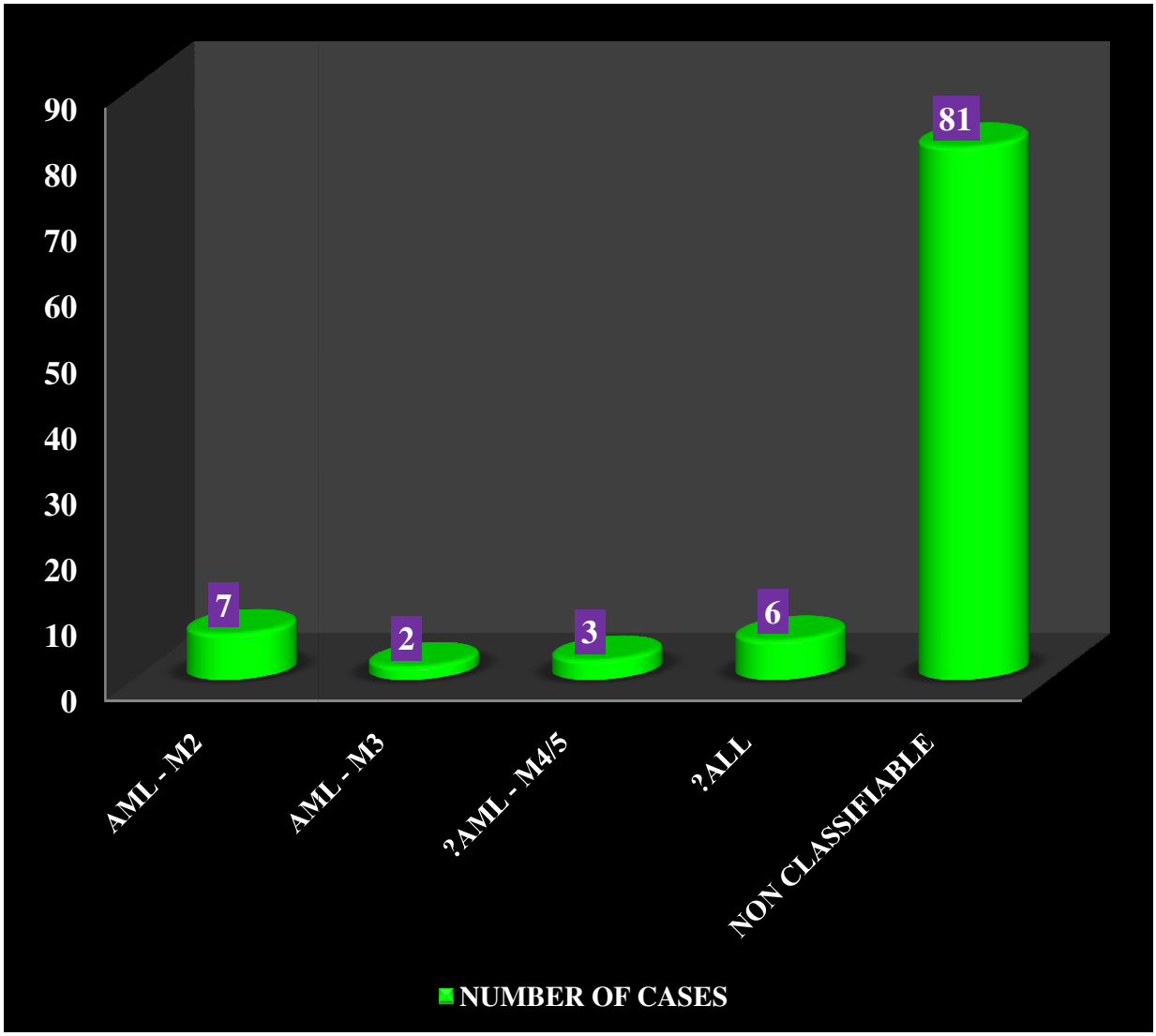
Figure 2: Sex distribution of acute leukemia



Test records of the 99 cases of acute leukemia were accessed. Bone marrow aspiration (BMA) study was performed on 47 of these cases. Bone marrow trephine biopsies were also formed on 40 of these 47 BMA cases (85%).

Of the 99 cases diagnosed as acute leukemia in the peripheral smear/ bone marrow aspirate/ both, a definitive diagnosis of the type of acute leukemia based purely on the morphology, was possible in only 9 cases (Figure 3). All of these were acute myeloid leukemia. 6 cases were suspected to be acute lymphoid leukemia requiring further studies for confirmation. 3 others were suspected to be acute myeloid leukemia – myelomonocytic/ monoblastic type (AML- M4/5). Thus only 9% of acute leukemias could be typed with certainty using morphology alone.

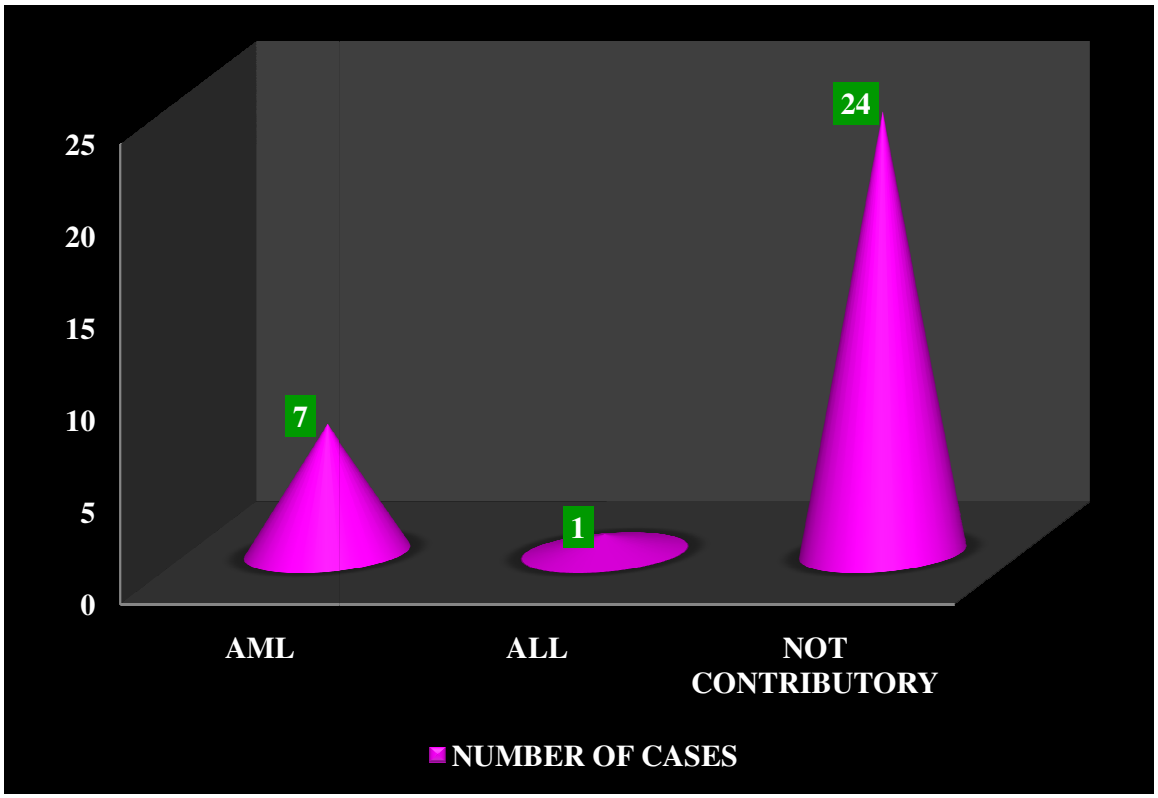
Figure 3: Distribution of types of acute leukemia reported on PS/BMA/both based only on morphology



Case records of the 81 cases diagnosed only as acute leukemia, showed that cytochemistry was done only in 32 of the cases, either on peripheral smears

or bone marrow aspiration smears. Of these, a definitive diagnosis on the type of leukemia was made on 8 cases (25%). Of these 8 cases, 7 were AML (myeloperoxidase positive) and 1 was diagnosed as ALL (block positivity with periodic acid Schiff). In the remainder, results of cytochemistry were declared as non – contributory with a request to perform ancillary tests. Figure 4 shows the results of cytochemistry performed on 32 of the cases of acute leukemia.

Fig 4: Distribution of types of acute leukemia following cytochemical studies



Of the 9 cases of AML diagnosed by morphology on Romanosky stained PS/BMA smears, cytochemistry was done in 7 cases. Of these, results of cytochemistry in 6 cases were corroborative with the morphological diagnosis i.e the blasts were positive for myeloperoxidase. On 1 case (AML – M2), cytochemistry was not contributory.

6 cases were suspected to be ALL (L1 orL2) on morphological grounds. Cytochemistry was done in all of them. In 3 cases, the lymphoblasts showed classical cytoplasmic block positivity for PAS confirming the diagnosis. PAS was negative in the other 3.

In 3 cases where a presumptive diagnosis of AML M4/M5 was offered, cytochemistry was done on all 3 cases. The blasts were negative for both MPO and PAS. Thus cytochemistry was not contributory in any of these cases. Table 15 is a summary of the results of cytochemistry. While only 9 cases could be diagnosed by morphology, an additional 11 cases could be typed using cytochemical studies with Myeloperoxidase and Periodic Acid Schiff stains.

Table 15: Summary of results of cytochemistry

Morphologic diagnosis on PS/BMA smears	Cytochemistry		Utility of results of cytochemistry (MPO & PAS)
	Done	Not done	
Acute leukemia	32	49	7 – diagnosed as AML 1 – diagnosed as ALL 24 – not contributory
AML	7	2	6 – corroborative 1 – not contributory
?ALL	6	0	3 – diagnosed as ALL 3 – not contributory
? AML M4/M5	3	0	Not contributory in all 3
Total	48	51	11 cases typed as AML or ALL 6 – corroborative with morphological diagnosis 31 – not contributory
Grand total	99		

Bone marrow trephine biopsy was performed in 40 of the total 99 cases of acute leukemia. Review of slides and blocks showed that 4 were inadequate for IHC studies. Hence immunohistochemistry was performed in 36 of the bone marrow trephine biopsies. Fresh sections from the blocks of all these cases were cut and immunohistochemistry was performed using four primary antibodies – Myeloperoxidase (MPO), CD20, CD3 and terminal deoxynucleotidase (TdT). Table 16 shows a breakup of the diagnostic

status of the study population prior to the application of IHC markers. We have considered all those cases speculated to be ALL or AML on morphology (with cytochemistry also being not contributory), into the broad category of acute leukemia.

Table 16: Diagnosis prior to the application of IHC studies

Diagnosis		No of cases	
Acute Leukemia		20	
AML	Diagnosis by morphology	6	11
	Diagnosis only after cytochemistry	5	
ALL	Diagnosis by morphology	0	5
	Diagnosis only after cytochemistry	5	
Total cases (study population for IHC studies)		36	

Of the 20 cases of acute leukemias, the IHC panel could type 18 of them as either myeloid or lymphoid (Table 17). Thus IHC has helped in subtyping 90% of the cases of acute leukemia. 1 case did not express any of the phenotypes studied. Another case showed positivity for both lineages.

Table 17: Subtypes of Acute Leukemia after IHC studies

Subtype of Acute Leukemia after IHC study	No. of cases
AML	11
ALL	7
Acute Leukemia of Ambiguous Lineage	1
Not contributory	1
Total	20

Figure 5 illustrates the number of acute leukemias that could be typed using morphology or cytochemistry or IHC using the study panel.

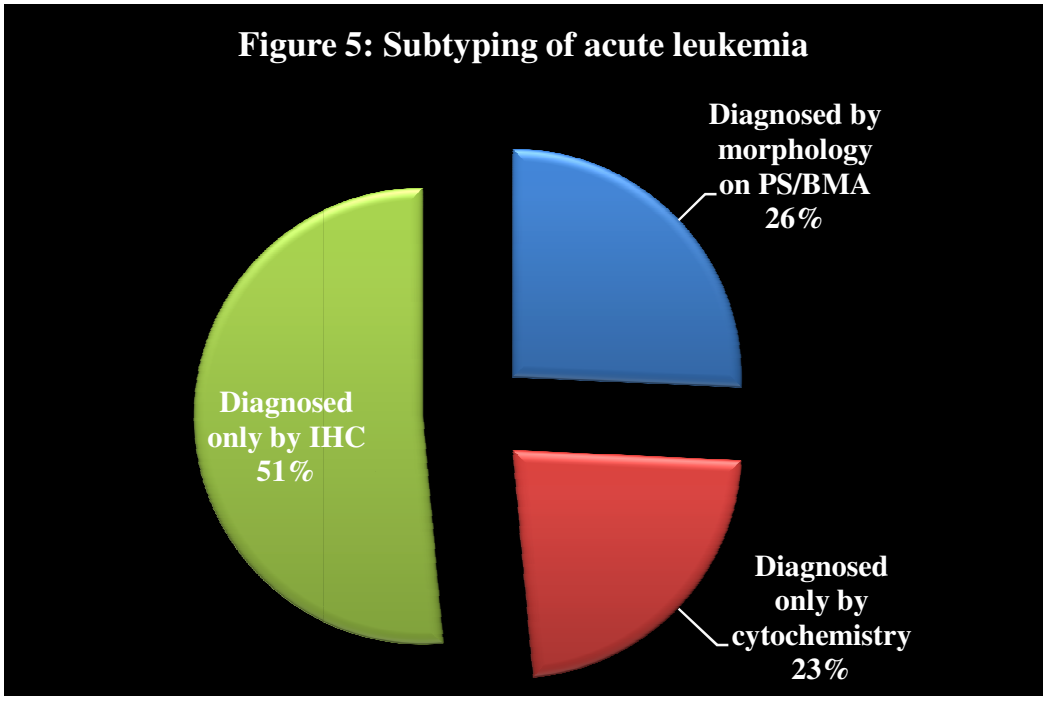


Table 18 shows that IHC had a 100% correlation with the subtype of acute leukemia that was diagnosed prior to IHC studies, either by morphology or cytochemistry. All the AML cases were positive for MPO and they were uniformly negative for CD20, CD3 and TdT. 4 of the ALL cases were positive for CD20, 1 was positive for CD3 and 3 were positive for TdT.

Table 18: Results of IHC on acute leukemia already typed by morphology or cytochemistry

Diagnosis	No of cases	MPO	CD20	CD3	TdT
AML	11	11/11	0/11	0/11	0/11
ALL	5	0/5	4/5	1/5	3/5

Table 19 shows the overall summary of the phenotypes of the immature cells after the performance of IHC studies. Of the ALL cases, 83% were of B cell lineage (ie CD20 positive) and TdT was positive in 90% of them. 17% of ALL were of T cell lineage and 1 of them was positive for TdT. None of the AML blasts were positive for TdT. Overall 31% of the acute leukemia was positive for TdT.

Table 19: Summary of the Phenotypes of Acute Leukemia.

Diagnosis	No of cases	MPO	CD20	CD3	TdT
AML	22	22/22	0/22	0/22	0/22
ALL	12	0/12	10/12	2/12	10/12
Acute leukemia of ambiguous lineage	1	1/1	0/1	1/1	1/1
Not contributory	1	0/1	0/1	0/1	0/1
All leukemias	36	23/36	10/36	3/36	11/36

PSG Hospitals, a tertiary care hospital, is a teaching affiliate of PSG Institute of Medical Sciences & Research, Coimbatore. The hospital caters to an average outpatient strength of 1060/day and an inpatient strength of 900/day.

The clinical pathology laboratory (CP Lab) receives samples from these patients and also from those attending the peripheral outreach clinics affiliated to the PSG Hospitals.

The hospital, as a policy, performs a few definitive screening investigations on all its new patients, which include Complete Blood Counts and Urine analysis. Hence they are the most common tests performed in the CP Lab. Peripheral smears are generally requested as a follow up after CBC tests. Hence they account only for 7% of the hematology tests. Bone marrow studies are performed most often, only upon the advice of the pathologist, except under certain situations (e.g. work up of lymphoma, myeloma, metastatic disease, pyrexia of unknown origin etc).

The CP lab maintains a register of all the peripheral smears reported by the pathologists. This register has the information on patient name, age, sex, IP/ OP number, referring unit/consultant, diagnosis and comments. This register was therefore useful in identifying all those peripheral smears which bore a

diagnosis of leukemia or were suspected to be leukemia with recommendations to perform ancillary tests such as bone marrow aspiration (BMA) and bone marrow trephine (BMT) biopsy.

PSG Hospitals has one of the advanced electronic data processing centers with well trained staff and facilities. The Hospital Information System (HIS), designed in – house, helped us to jot down results of all the information on the tests relevant to our study i.e. cytochemistry, BMA and BMT (hematoxylin & eosin stained section studies).

We observed that during the study period, the CP lab had reported 344 cases of leukemia, all types inclusive. Thus on an average, 76 new cases of leukemia were reported each year. This is significantly higher than the observation made by D' Costa GG et al, where they found an annual occurrence of about 24 cases over a 10 year period.[46]

Non – Hodgkin lymphoma and leukemia are listed in the top 10 leading sites of cancer in many centers that have contributed to the development of an Atlas of Cancer in India. [47] Xie Y, et al published their observations on the trends in leukemia in USA, over a period of 25 years (1973 - 1998). They noted that there was an overall increase in occurrence of leukemia in people lesser than 20 years old, most of which was due to an increase in the

incidence of ALL. However, the number of cases of leukemia in people older than 20 years decreased, chiefly due to a decline in the incidence of CML and CLL. There was no change in the incidence of AML. [48]

Acute leukemias comprised 28.7% of all leukemias in our study. This is much lesser compared to D'Costa et al's observations, where they comprised 58% of all leukemias. Thus there appears to be an obvious geographical variation in the pattern of leukemias within India.

The peak age of occurrence of acute leukemia which showed a bimodal pattern compared well with D' Costa et al's study and Anuradha Kusum et al's study. [49]

While our study showed only a slight male preponderance (M:F = 1.3:1) in the sex incidence of acute leukemias, a higher occurrence in men was noted in D' Costa et al and Anuradha Kusum et al studies i.e. 2.7:1 and 2.3:1, respectively.[46],[49]

All acute leukemias are required to be classified either as AML or ALL. This is crucial for 2 interdependent reasons – one, to choose the most appropriate ancillary investigation for exact sub typing and second to offer the most appropriate therapy. Ancillary tests are either cytogenetic studies or flow cytometry or a combination of both. The advent of targeted gene

therapy has made it imperative for this sub typing to be done. Parenthetically, as ancillary tests are very expensive, unless one is sure about the blast lineage, specific ancillary tests cannot be asked for.

Our study showed that a definitive sub typing of acute leukemia as AML or ALL, purely by morphology, could be made in only 9 of the cases. The CP lab used only two cytochemical markers (viz myeloperoxidase and periodic acid Schiff), in the cases wherever cytochemistry was performed. Sudan black B was used infrequently. Hence it was not used in our data collection tool. Usage of these two cytochemical markers had helped in the typing of 8 more cases of acute leukemia. Thus, of the 99 cases of acute leukemia, only 17 cases could be sub typed as AML or ALL on morphology and cytochemistry. 14 of these 17 cases of acute leukemia were diagnosed as acute myeloid leukemia because, in all of these cases, the myeloblasts showed diagnostic Auer rods which are crystalline structures seen only in AML or in high grade myelodysplastic syndrome. They are never seen in lymphoblasts. [50] All the three cases of ALL could only be diagnosed after cytochemistry where the PAS reaction showed block positivity. Loffler H et al opine that ALL diagnosed by cytochemistry must be confirmed by immunophenotyping in all the cases. [51]

The FAB system which relied solely on morphology and cytochemistry continues to be used for the preliminary morphological assessment, but clearly, this is not enough. The recent WHO classification of hematopoietic neoplasms therefore relies extensively on immunophenotyping and of cytogenetic and molecular genetic analysis. [20]

3 cases during the study period were diagnosed empirically as AML M4/M5. Review of the slides of these cases showed a preponderance of monoblasts and its derivatives with scarce myeloblasts. The monoblasts were large with abundant cytoplasm and an almost round nucleus. Faint granules and vacuolations were seen in some of them. All the 3 cases were negative for cytochemistry with myeloperoxidase and PAS. It would have been prudent if the cytochemistry panel included non specific esterases such as alpha napthyl butyrate (ANB) and alpha napthyl acetate (ANA) as they would have helped in the confirmation of monoblasts, promonocytes and monocytes. The WHO recommends a combination of 2 esterases in such cases, such as any one of the 2 non – specific esterases and 1 specific esterase such as naphthol – ASD – chloracetate esterase, so that the former would identify monocytic lineage cells and the latter, neutrophil lineage cells.

Using the HIS software, we had observed that in only 40 of the 99 cases of acute leukemia, was a bone marrow trephine biopsy done. On reviewing the slides 4 were excluded. 2 of them showed cartilage, cortical bone and sub cortical space only. Trabecular bone and marrow spaces were not seen. The other 2 cases had blood clots and cutaneous tissue only. Hence the final study population i.e. cases diagnosed as acute leukemia and had trephine biopsy blocks available for IHC studies, was 36.

Immunophenotyping of the leukemic blasts is essential for the diagnosis and confirmation of acute leukemia into 1 of the 2 types (AML or ALL). Immunophenotyping refers to the identification of specific characteristic nuclear, cytoplasmic or cell surface antigens expressed by the leukemic cells. This is achieved by the recognition of these specific antigens by labeled antibodies.

The detection system can either be flow cytometry or immunohistochemistry. Till recently, flow cytochemistry was preferred over immunohistochemistry, because it could be performed quickly and allows a more precise quantification of the percentage of positive cells. The same cell could express many types of antigens and these when bound by appropriate labeled antibodies, could be identified by forward and sideward

light scatter. [52] However, flow cytometry is very expensive, out of reach for the common man and technically non-feasible to be performed on archived tissues. IHC on the other hand, is a two dimensional system and generally involves application of 1 specific antibody per section. However, current IHC systems use multicolor combinations and 3 to 5 different antigens can be identified on the same section.

Paraffin section IHC is a simple tool requiring skilled personnel and very little of equipment. Immunophenotyping of leukemic cells on tissue sections used to be infamous because of lack of consistent results. However, in the late 1990s there had been major advancements in the techniques of IHC, especially on the unmasking of antigenic epitopes on the paraffin tissue sections. This, coupled with the increased availability of commercial antibodies for lineage determination, has resulted in the resurgence of the use of IHC on bone marrow trephine tissues. [30] The WHO in its most recent issue on classification system, acknowledges that IHC can be used as a diagnostic tool for immunophenotypic analysis. [20]

Literature is chocked with information on various IHC panels that can be used in the sub typing of acute leukemia. Tables 8 to 10 summarize the various markers that can be used to diagnose AML and ALL, including their

subtypes. However, it is prudent that every laboratory develops its own inventory of IHC panel markers keeping in mind the commonality of types and finances involved.

Anti MPO, CD13 and CD33 are the 3 IHC markers used consistently to detect blasts of myeloid lineage, by the 3 major conglomerates in hematology namely, European Group for the Immunological characterization of Leukemias (EGIL), US – Canadian Consensus Group (USCC) and British Committee for Standards in hematology (BCSH).[52] Of these CD13 and CD33 are best detected by flow cytometry. Anti MPO, however is best detected by IHC and has a very high specificity for myeloid lineage. [53] Hence anti MPO was the first to be included in our panel. We were aware that AML-M5 may not be picked up. However AML-M5 is rarely reported in our centre and monoblasts have a morphology distinct from myeloblasts.

CD19, CD20, CD10 are the 3 markers common to all the 3 panels (EGIS, USCC & BCSH) for the detection of lymphoblasts of B cell type. CD19 is commonly used in Flow Cytometry and is not preferred for IHC studies as reliability tests are incomplete.[28] CD10 can be detected both by flow cytometry and immunohistochemistry. However a proportion of normal

hematogones also express CD10. [52] Hence CD20 was the 2nd marker to be included into our panel.

CD 2 is the only IHC marker that is found in all the 3 panels of the expert groups, to detect lymphoblasts of T cell lineage. CD3 and CD7 are common to 2 systems. Hence we had to choose between CD2, CD3 and CD7. Chaung SS et al observed that anti CD3 specifically stained T lymphocytes on paraffin sections. [53] They also noted that it was non-reactive with B lymphocytes, granulocytes and non hematopoietic tissues. Hence CD3 was the 3rd marker to be included in our panel.

The last to be included in our panel was TdT. This is a marker of immature hematopoietic and lymphoid cells. It is not lineage specific. It is positive in many of the ALL (B subtype in particular) and a few cases of AML (AML M0, M1). [52]

Prior to the application of IHC on tissue sections, 20 of the 36 cases of the study population were acute leukemia i.e., they could not be typed as myeloid or lymphoid. However, 18 of them could be clearly categorized as either AML or ALL after the application of our IHC panel ***(Colour plates 1 & 2)***. Thus the lineage of 90% of acute leukemia could be identified. This compares well with the observations made by Arber DA et al who noted that

96% of acute leukemias could be sub typed effectively. However, their IHC panel included CD34, CD43, CD68, CD79a and HLA – DR in addition to the four used by us. The authors also observed that the lineage of the blasts could still be determined accurately even when the results are extrapolated to 4 specific IHC markers viz., CD3, CD79a, MPO and TdT. The authors conclude their paper noting that, if CD20 replaced CD79a, it could pick up rarer cases of ALL which are negative for CD79a and are positive for CD20. [30]

A similar study was performed at the Kidwai Memorial institute of Oncology located at Bangalore. In addition to the 4 markers used in our study, they had also used CD68 and F8RA. Even in this study, the authors have observed that a limited panel of the 4 markers (as was used in our study) was effective enough to type acute leukemia as AML or ALL. They also noted that this panel has a distinct cost advantage compared to flow cytometry.

1 case of acute leukemia expressed both myeloid and lymphoid phenotypes i.e. MPO, CD3 and TdT. The recent WHO classification of hematopoietic tumors acknowledges a broad entity called acute leukemias of ambiguous lineage which include all those acute leukemias that do not express any

phenotype of differentiation and those that express antigens of more than 1 lineage to such an extent that it may not be possible to assign any 1 lineage with certainty. Acute leukemias which express antigens of more than 1 lineage are also termed as mixed phenotype acute leukemias (MPAL). These are rare and account for 4% of all cases of acute leukemia. [54] The occurrence of MPAL in our study population was 3% which is similar to that reported in literature.

Results of all the 11 cases of AML and 5 of ALL that were diagnosed by morphology and cytochemistry corroborated well with IHC studies ***(Colour plates 3, 4 & 6)***. A much larger correlative study done by Browman GP et al showed a concordance of 99%. The authors opine that even though a morphological diagnosis of the type of acute leukemia is made, IHC has to be done for confirmation, as morphological assessment has a high inter-observer discordance. [55]

All the 3 cases which were suspected to be AML – M4/M5 and were found to be negative for cytochemistry, turned out to be AML on IHC ***(Colour plate 5)***. MPO was expressed randomly in about 20 – 40% of the immature cells. The remainder was negative for MPO and to the other 3 IHC markers of our study. These could be monoblasts and their derivatives. CD68 detects

them by flow cytometry. However it is not effective in IHC as all FAB types of AML are reported to express this antigen. Antibody against lysozyme may be useful in such cases. [30]

1 case of acute leukemia was negative for all the 4 IHC markers used in our study. 4 types of acute leukemia can classify for this presentation. They are AML with minimal differentiation, acute monoblastic leukemia, acute erythroid leukemia and acute megakaryoblastic leukemia. Of these, TdT may occasionally be positive in AML with minimal differentiation and Acute megakaryoblastic leukemia. We reviewed the morphology and results of cytochemistry of this case. The blasts showed no evidence of differentiation and had very scanty, agranular deeply basophilic cytoplasm. Prominent nucleoli, numbering 1 to 4 were also seen. This morphology clearly ruled out the possibility of AML – M5 as monoblasts have a distinct morphology. The absence of irregular or indented nuclear contours and cytoplasmic blebs and pseudopods were against the typical morphological appearance of a megakaryoblast. Results of cytochemistry showed that the cytoplasm was negative for a PAS reaction. Hence pure erythroid leukemia is also unlikely. Therefore it is possible that, this is a case of AML with minimal differentiation. Upto 50% of cases of AML with minimal differentiation are negative for TdT. Hence the only way to confirm that this

is a case of AML with minimal differentiation is to test for CD34, CD38 and HLA – DR.

1. On an average, 76 new cases of leukemia are diagnosed each year in the clinical pathology laboratory of PSG Hospitals. Of these 28.7%, are acute leukemias.

2. Acute leukemias had a bimodal peak age of occurrence i.e. 0 – 20 years and 41 – 60 years.

3. Acute leukemias had a mild male preponderance (M: F=1.3:1). This contrasts from other published data from Bombay and North India which showed a much higher male predominance (M: F=2.3 to 2.7:1).

4. Only 9 cases of acute leukemia could be typed as AML or ALL purely by morphology. Another 10 cases could be typed using cytochemistry.

5. A limited immunohistochemical panel of MPO, CD20, CD3 and TdT used in our study helped to type 90% of cases of acute leukemia

(where bone marrow trephine biopsy was performed) into AML or ALL.

6. Results of all the acute leukemias that were diagnosed by morphology or cytochemistry had a 100% correlation with the results of immunohistochemistry.

7. The IHC panel used also identified 1 case of AML of ambiguous lineage while another was negative for all the markers.

8. Based on our results, we suggest the use of this limited panel of immunohistochemical markers that includes MPO, CD20, CD3 and TdT, for the routine evaluation of all acute leukemias in paraffin embedded tissues. For a resource poor country such as ours, it is easier to type acute leukemia using immunohistochemistry than by flow cytometry given the disadvantage of the costs involved with the latter.

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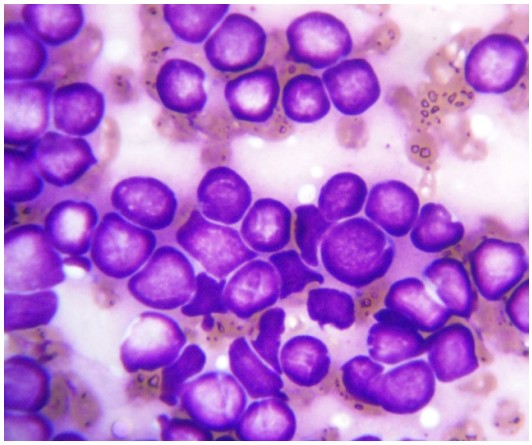
Master Chart of cases where Immunochemistry study was performed.

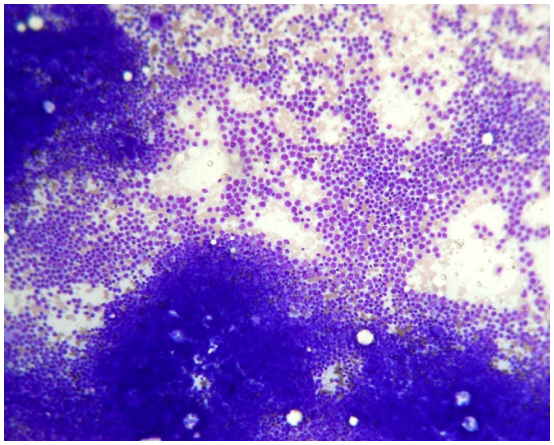
S. NO	AGE/ SEX	IP/OP NUMBER	PS & BMA MORPHOLOGY REPORT	CYTOCHEMISTRY		IMMUNOHISTOCHEMISTRY			
				MPO	PAS	MPO	CD20	CD3	TdT
1	48/F	I08003476	Acute leukemia	+	-	+	-	-	-
2	51/M	I08014000	Acute leukemia	+	-	+	-	-	-
3	5/M	I08015070	? ALL/ L1	-	+	-	+	-	+
4	51/F	I08017278	AML M3	-	+	+	-	-	-
5	45/M	I08019864	? ALL / L2	-	+	-	+	-	-
6	52/F	I08041085	Acute leukemia	Not contributory		-	-	+	-
7	81/M	I08019401	? ALL	-	+	-	-	+	+
8	31/M	I08038009	Acute leukemia	Not contributory		+	-	-	-
9	75/F	I09035118	AML M2	+	-	+	-	-	-
10	18/M	I09040507	? ALL / L2	Not contributory		-	+	-	+
11	45/M	I09000925	? ALL/ L2	-	+	-	+	-	+
12	51/M	I09015241	Acute leukemia	Not done		+	-	-	-
13	57/F	I09002317	? AML M4/M5	+	-	+	-	-	-
14	14/M	I09032945	Acute leukemia	Not contributory		-	+	-	-
15	1/M	I09041399	Acute leukemia	Not contributory		-	+	-	+
16	19/M	I09000046	Acute leukemia	Not done		+	-	-	-
17	3/F	I09022081	Acute leukemia	Not contributory		-	+	-	+
18	45/F	I10000926	Acute leukemia	Not done		+	-	-	-
19	24/F	I10003606	Acute leukemia	+	-	+	-	-	-
20	41/M	I10005666	Acute leukemia	+	-	+	-	-	-
21	39/M	I10006895	AML M3	+	-	+	-	-	-
22	45/M	I10032796	AML M2	Not contributory		+	-	-	-
23	14/M	I10001284	Acute leukemia	Not contributory		-	-	-	-
24	32/F	I10017006	Acute leukemia	Not contributory		+	-	-	-
25	45/F	I10035882	? ALL / L2	-	+	-	+	-	-
26	65/M	I10036764	Acute leukemia	Not contributory		+	-	-	-
27	65/M	I11003513	Acute leukemia	+	-	+	-	-	-

Master Chart of cases where Immunochemistry study was performed.

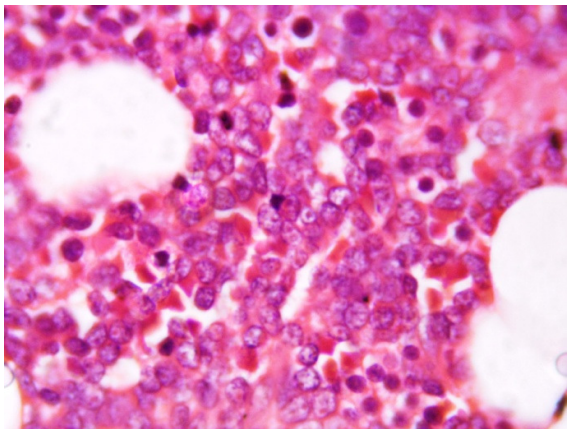
S. NO	AGE/ SEX	IP/OP NUMBER	PS & BMA MORPHOLOGY REPORT	CYTOCHEMISTRY		IMMUNOHISTOCHEMISTRY			
				MPO	PAS	MPO	CD20	CD3	TdT
28	15/M	I11006102	Acute leukemia	-	+	-	+	-	+
29	74/M	I11001123	? AML M4/M5	Not contributory		+	-	-	-
30	15/M	I11018027	Acute leukemia	Not contributory		-	+	-	+
31	58/F	I11027567	Acute leukemia	Not contributory		+	-	-	-
32	54/M	I11030526	? AML M4/M5	Not contributory		+	-	-	-
33	22/F	I11029963	Acute leukemia	Not done		+	-	-	-
34	47/F	I12004086	Acute leukemia	Not contributory		+	-	-	-
35	44/M	I12011269	Acute leukemia	Not contributory		+	-	+	+
36	21/F	I12015708	Acute leukemia	+	-	+	-	-	-



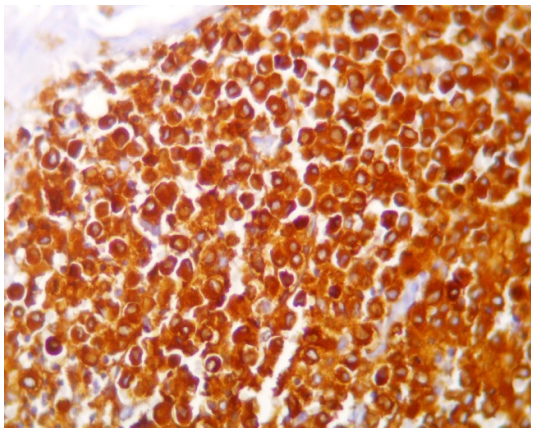
Peripheral smear reported as acute leukemia as there were no differentiating features; Leishman; x1000



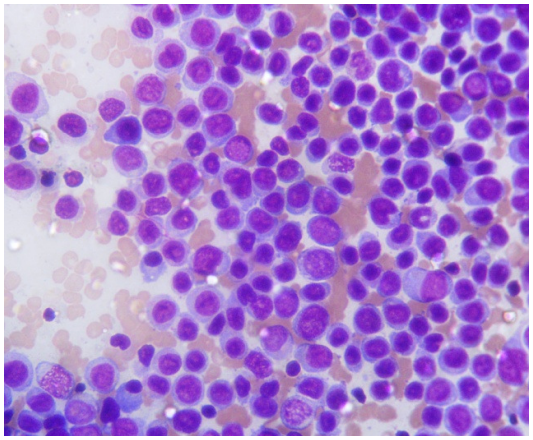
Hypercellular bone marrow aspirate smear composed of a monotonous population of undifferentiated blasts; Giemsa; x100



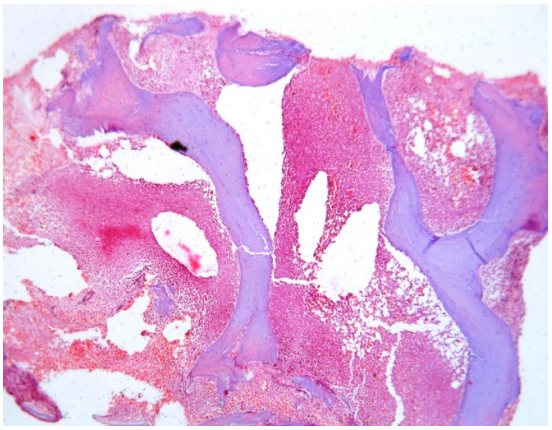
Bone marrow trephine biopsy showing hypercellular marrow spaces replaced by undifferentiated blasts; H&E; x400



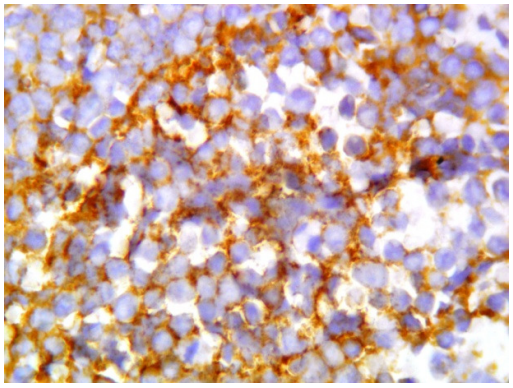
Blasts are diffusely positive for myeloperoxidase on IHC; x400



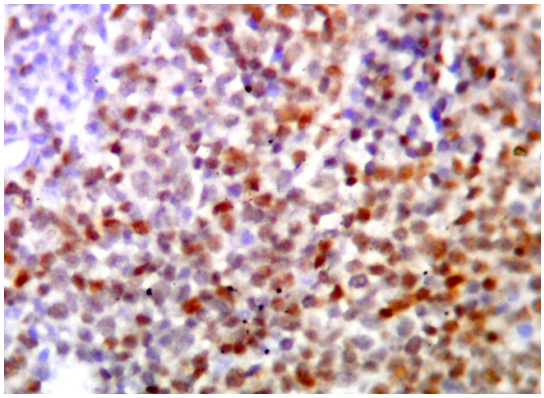
Bone marrow smear reported as acute leukemia; Giemsa; x400



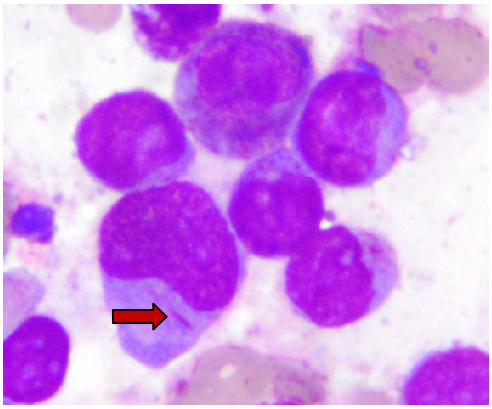
Bone marrow section showing extensive replacement of marrow spaces by blasts; H&E; x40



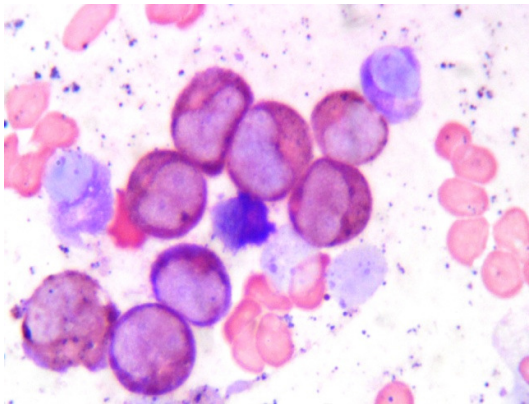
Blasts showing positivity for CD 20 by IHC; x400



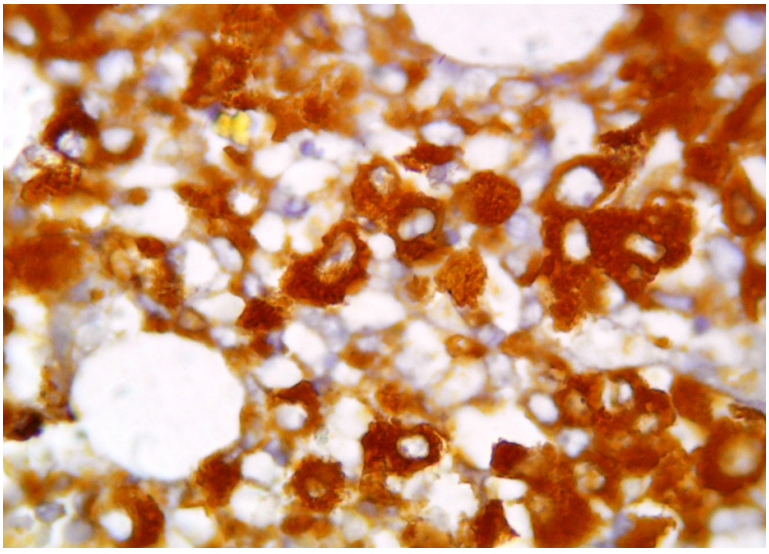
Blasts are also positive for TdT by IHC; x100



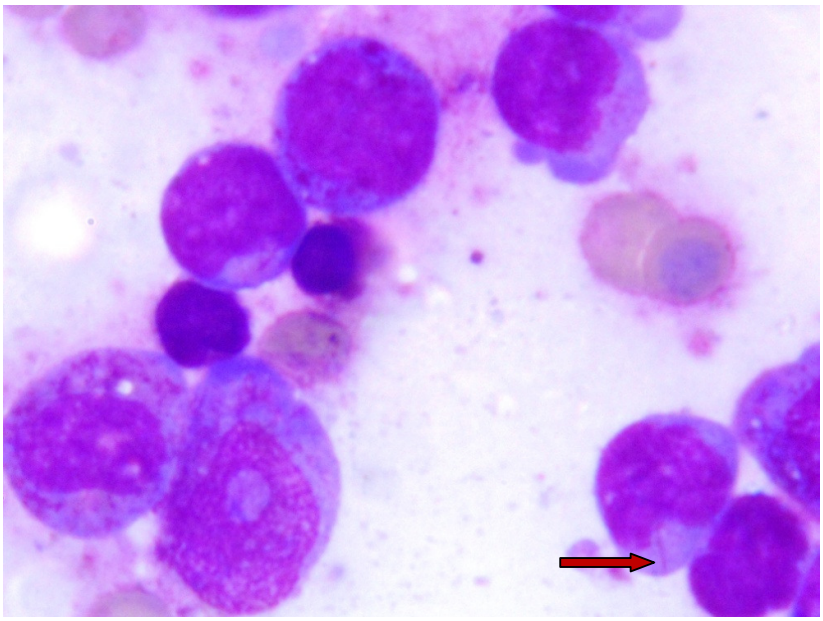
Peripheral smear reported as AML-M2 with an Auer rod in a myeloblast (arrow); Leishman; x1000



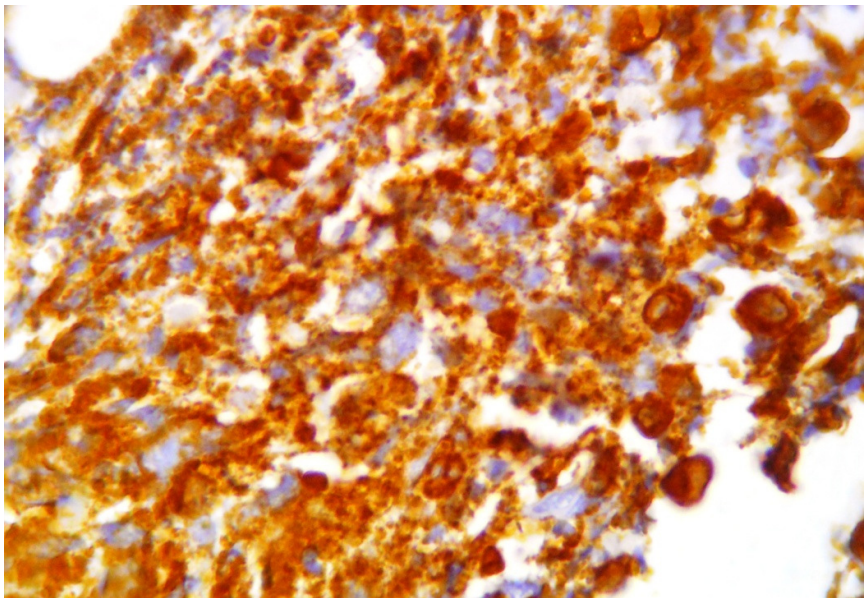
Myeloblasts showing cytoplasmic granular positivity for myeloperoxidase; x400



Myeloblasts staining intensely positive for myeloperoxidase on IHC study; x1000



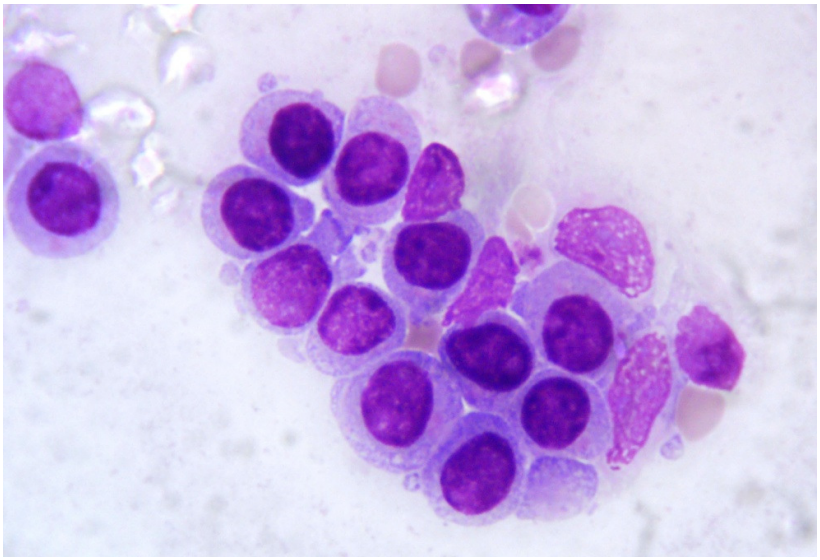
Bone marrow smear reported as AML-M3 showing many promyelocytes and a myeloblast with an Auer rod (arrow); Giemsa; x1000.



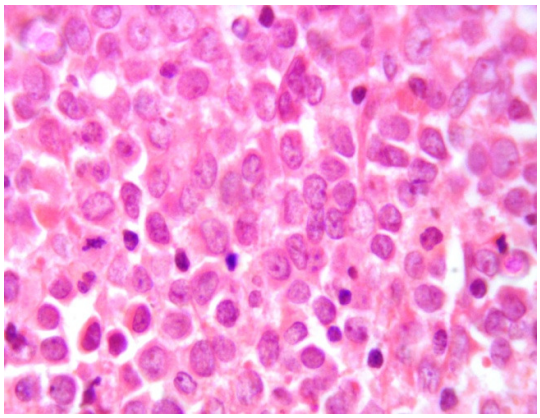
Myeloblasts staining intensely positive for myeloperoxidase on IHC study; x1000

A case of acute leukemia suspected to be AML-M4/M5, diagnosed as AML by immunohistochemistry

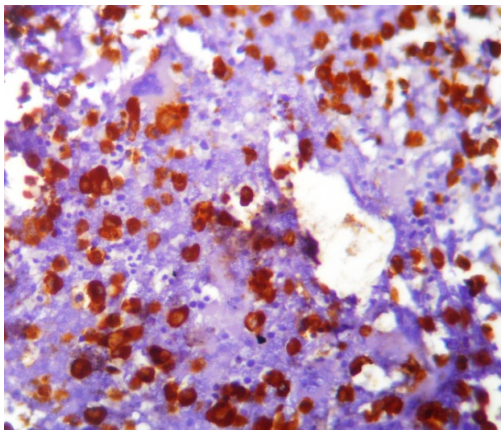
Color plate 5



Bone marrow smear reported as ?AML-M_4/M_5 showing many monoblasts with abundant cytoplasm and an occasional promonocyte; Giemsa; x1000. Myeloblasts were also seen elsewhere.



Bone marrow trephine biopsy showing numerous blasts with abundant cytoplasm and occasional promonocytes with prominent nuclear folds; H&E; x1000



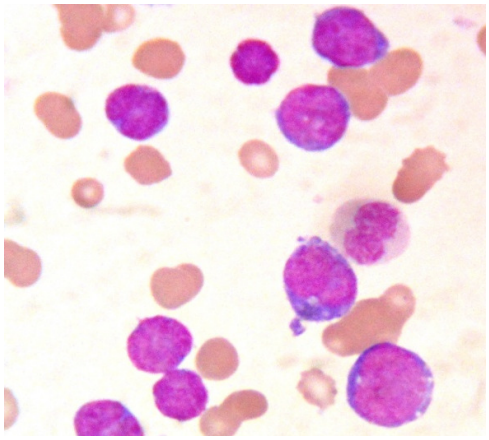
Some of the blasts are positive for myeloperoxidase on IHC, indicating that they are myeloblasts. Others are negative. These could be monoblasts and promonocytyes; x400

A case of acute leukemia suspected to be AML-M4/M5, diagnosed as AML by immunohistochemistry

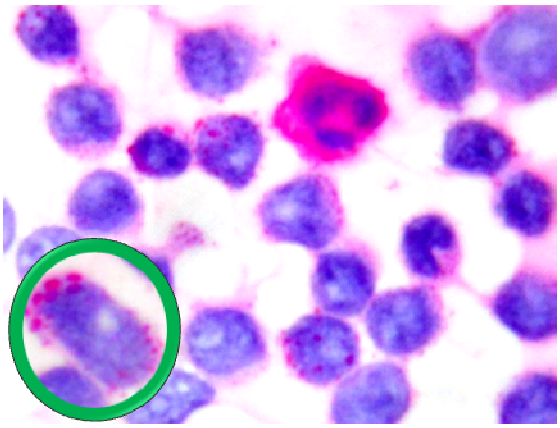
Color plate 5

A case of acute leukemia suspected to be ALL-L2, diagnosed with cytochemistry and confirmed by IHC

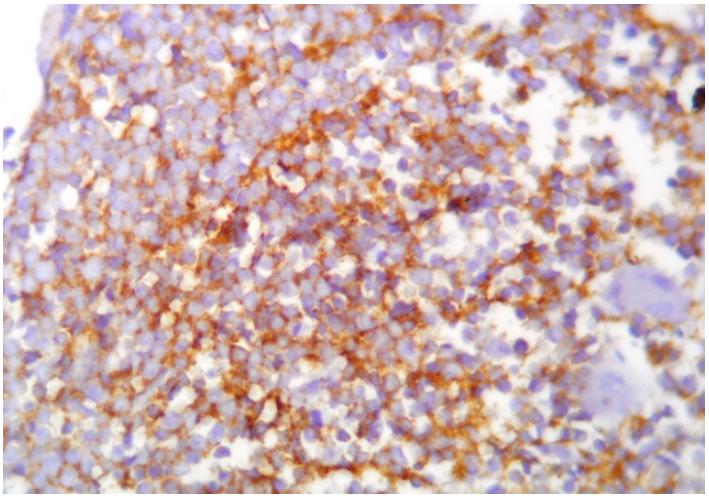
Color plate 6



Peripheral smear reported as ?ALL-L2. Blasts showing variation in size, occasional nucleoli and variable amounts of cytoplasm; Leishman; x400



Blasts showing typical block positivity with PAS stain. The cytoplasm of the neutrophil which stains diffusely positive, serves as an internal control; x400. Inset shows lymphoblast with large magenta colored blocks; PAS; x 1000.



Lymphoblasts showing positivity for CD20 on IHC study ; X 400